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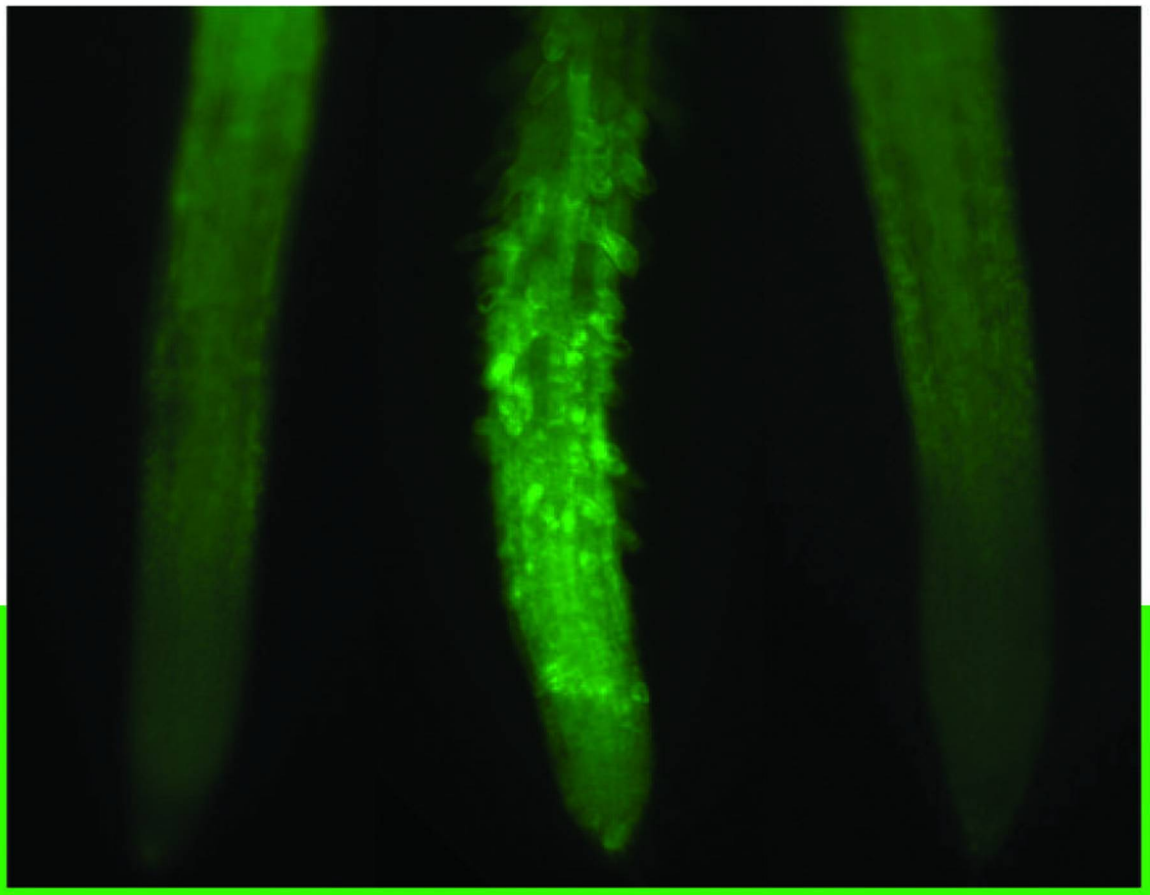

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ARTICLE

Exogenous ascorbic acid is a pro-nitrant in *Arabidopsis thaliana*

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ABSTRACT Due to the intensified production of reactive nitrogen species (RNS) proteins can be modified by tyrosine nitration (PTN). Examination of PTN is a hot topic of plant biology, especially because the exact outcome of this modification is still pending. Both RNS and ascorbic acid (AsA) are redox-active molecules, which directly affect the redox state of cells. The possible link between RNS-dependent PTN and AsA metabolism was studied in RNS (*gsnor1-3*, *nia1nia2*) and AsA (*vtc2-3*) homeostasis *Arabidopsis* mutants. During physiological conditions, intensified PTN was detected in all mutant lines compared to the wild-type (WT); without altering nitration pattern. Moreover, the increased PTN seemed to be associated with endogenous peroxynitrite ($ONOO^-$) levels, but it showed no tight correlation with endogenous levels of nitric-oxide (NO) or AsA. Exogenous AsA caused intensified PTN in WT, *vtc2-3* and *nia1nia2*. In the background of increased PTN, significant NO and $ONOO^-$ accumulation was detected, indicating exogenous AsA-induced RNS burst. Interestingly, in AsA-triggered stress-situation, changes of NO levels seem to be primarily connected to the development of PTN. Our results point out for the first time that similarly to human and animal systems exogenous AsA exerts pro-nitrant effect on plant proteome. **Acta Biol Szeged 62(2):115-122 (2018)**

KEY WORDS
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Introduction

On the analogy of oxidative stress, the concept of nitrosative stress became widely accepted in the last years. The group of molecules responsible for nitrosative stress - called reactive nitrogen species (RNS) – contains nitric oxide (NO), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), S-nitrosoglutathione (GSNO), S-nitrosothiols (RSNO), nitrogen dioxide (NO_2) or nitrosonium cation (NO^+) (Wang et al. 2013). In order to fulfil their role, they might co-interact with different signal molecules (e.g., MAPK cascade, cGMP, Ca^{2+}), or they are also able to directly modify proteins, fatty acids and presumably nucleic acids (Patel et al. 1999).

Tyrosine nitration, a posttranslational modification of proteins means the addition of a nitro group ($-NO_2$) to one of the two equivalent *ortho* carbons in the aromatic ring of the tyrosine amino acids (Gow et al. 2004). In this process, $ONOO^-$ plays an important role as the precursor of molecules chemically responsible for PTN itself (Yeo et al. 2015; Radi 2012). Peroxynitrite is formed in the reaction between superoxide anion ($O_2^{\cdot-}$) and NO at the production sites of $O_2^{\cdot-}$ (Denicola et al. 1998). PTN might affect the function and fate of a protein in different ways: beside no effect on the function (Begara-Morales et al. 2015), in most cases PTN results in the inhibition of protein activity (Greenacre and Ischiropoulos 2001; Radi 2004).

The result of PTN is mostly examined in stressed plants, in connection with the appearance of nitro-oxidative stress (Corpas et al. 2007; Mata-Pérez et al. 2016). Beyond stress-induced nitration, evidences suggest that PTN might happen during physiological conditions as well, which means that a part of the proteome is being nitrated even under control circumstances (reviewed by Kolbert et al. 2017). Furthermore, most of the results are obtained in *Arabidopsis* and crop plants, while we still have very little knowledge about the nitroproteome of mutant *Arabidopsis* lines.

Generation and different impacts of reactive oxygen species (ROS) dates back to the formation of oxygen-rich environment. High levels of ROS have the ability to damage macromolecules; hereby their concentration needs to be strictly controlled by the complex mechanisms of enzymatic- and non-enzymatic antioxidant systems (Apel and Hirt 2004). One of the most important non-enzymatic antioxidants is ascorbic acid (AsA) which is able to directly scavenge some of the ROS ($O_2^{\cdot-}$, singlet oxygen, hydroxyl radical, hydrogen peroxide (H_2O_2)) (Padh 1990); while through the activity of ascorbate peroxidase (APX) it participates indirectly in the elimination of H_2O_2 as well

(Foyer and Halliwell 1976; Asada 1992).

In plants exposed to environmental stresses, exogenous application of AsA has positive effects (Athar et al. 2009, Chao and Khao 2010). On the other hand, there is only limited data available about the effect of external AsA on healthy, unstressed plants and these studies reported pro-oxidant effects of exogenously applied AsA (Tyburski et al. 2012; Qian et al. 2014). Similarly, in human system, exogenously applied antioxidants, like AsA were shown to possess pro-oxidant property but besides, pro-nitrant effects have also been described (Bouayed and Bohn 2010).

The main goal of this study was to investigate the possible – but so far unknown – pro-nitrant (PTN-inducing) effect of exogenously applied AsA in a plant system. Also, the poorly known connection between physiological PTN and endogenous AsA levels has been examined using mutant *Arabidopsis thaliana* lines.

Materials and methods

Plant material and growth conditions

During the experiments, fourteen-day-old wild-type (WT, Col-0) and mutant *Arabidopsis thaliana* L. plants were used.

The *gsnor1-3* plants possess reduced S-nitrosoglutathione reductase (GSNOR) activity and higher total S-nitrosothiol, nitrate and NO levels (Feechan et al. 2005; Rustérucci et al. 2007; Lee et al. 2008). The *nia1nia2* mutant has a point mutation in NIA1 and a deletion in NIA2 gene, having only 0.5% of the nitrate reductase (NR) enzyme activity of the WT (Wilkinson and Crawford 1993). The *vtc2-3* contains 40-50% of the WT AsA level, caused by a mutation in VTC2 gene, responsible for GDP-L-galactose phosphorylase synthesis (Conklin 2001). All *Arabidopsis* lines had *Columbia* (*Col*) ecotype background.

The seeds of all plant lines were surface sterilised with 70% (v/v) ethanol followed by 5% (v/v) sodium hypochlorite and transferred to half-strength Murashige and Skoog medium (1% (w/v) sucrose and 0.8% (w/v) agar) (Murashige and Skoog 1962). In case of external AsA supply (100 and 500 μM), autoclaved agar medium was cooled to approximately 35 °C before the addition of AsA in order to avoid heat-caused degradation. Moreover, the pH of the medium was adjusted to 7 instead of the normal 5.7-5.8, to avoid its acidification after AsA supplementation.

The petri dishes were kept in a greenhouse at a photo flux density of 150 μmol m^{-2}/s (12/12 day/night period) at a relative humidity of 55-60% and 25 ± 2 °C.

Determination of AsA

250 mg plant material was grounded in 1 ml 5% (w/v) trichloroacetic acid (TCA) during sample preparation. The amount of total ascorbate was determined by the reduction of dehydroascorbate to ascorbate by dithiothreitol (DTT); the reduced AsA samples contained water instead of DTT. Ascorbate concentrations were determined spectrophotometrically at 525 nm and are expressed in μmol/g fresh weight. Dehydroascorbate content was calculated as the difference between total and reduced AsA concentration (Law et al. 1983).

Detection of NO, $ONOO^-$ and $O_2^{\cdot-}$ by fluorescent microscopy

For the detection of NO 1,2-diaminoanthraquinone (DAQ) was used (Seligman et al. 2008). Seedlings were incubated in 50 μM DAQ solution prepared in ultrapure water for 30 min at room temperature followed by a single washing step in water prior microscopic analysis. As control experiment NO donor sodium nitroprusside (SNP) was used (200 μM), while as NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; 800 μM) was applied; both SNP and cPTIO were applied for one hour at 150 μmol m^{-2}/s light intensity.

The content of $ONOO^-$ in the root tips was detected using dihydrorhodamine (DHR) staining (Sarkar et al. 2014). Roots were incubated in 10 μM DHR solution prepared in Tris-HCl (10 mM, pH 7.4), and were washed twice with Tris-HCl buffer. For testing the response of DHR to $ONOO^-$ and H_2O_2, *Arabidopsis* plants were pretreated with peroxynitrite donor 3-morpholinosydnonimine hydrochloride (SIN-1; 1000 μM; 1 h) or H_2O_2 (100 μM; 30 min).

Dihydroethidium (DHE) was used for visualisation of superoxide anion. Root tips were incubated in 10 μM dye solution for 30 min in darkness at 37 °C and were washed twice with TRIS-HCl buffer (10 mM, pH 7.4) (Kolbert et al. 2012).

The root tips of *Arabidopsis* plants stained with different fluorophores were investigated under a Zeiss Axiovert 200M inverted microscope (Carl Zeiss, Jena, Germany) equipped with a high resolution digital camera (Axiocam HR) and filter set 9 (exc.: 450-490 nm; em.: 515- ∞ nm) for DHE, filter set 10 (exc.: 450-490; em.: 515-565 nm) for DHR, filter set 20HE (exc.: 546/12; em.: 607/80) for DAQ. Fluorescence intensities (pixel intensity) in the meristematic zones of the primary roots were measured on digital images using Axiovision Rel. 4.8 software within circles of 100 μm radii.

Preparation of protein extract, SDS-PAGE and western blotting

Plant material was grounded with double volume of extraction buffer (50 mM Tris-HCl buffer, pH 7.6-7.8, containing 0.1 mM EDTA (ethylene diamine tetra acetic acid), 0,1% Triton X-100 (polyethyleneglycol p-(1,1,3,3-tetra-methylbutyl)-phenylether) and 10% glycerol. After

20 min centrifugation on 4 °C at 12 000 rpm the supernatant was stored at -20 °C. Protein concentration was determined using the Bradford assay (Bradford 1976) with bovine serum albumin as standard.

Protein extracts (30 μg per lane) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% acrylamide gels. For western blot analysis, separated proteins were transferred to PVDF membranes using the wet blotting procedure (30 mA, 16 h). After transfer, membranes were used for cross-reactivity assays with rabbit polyclonal antibody against 3-nitrotyrosine diluted 1:2000 (Corpas et al. 2008). Immunodetection was performed by using affinity isolated goat anti-rabbit IgG-alkaline phosphatase secondary antibody in dilution of 1:10 000, and bands were visualised by using NBT/BCIP reaction. As a positive control nitrated bovine serum albumin (NO_2-BSA) was used.

Statistics

The results are expressed as mean ± SE. Multiple comparison analyses were performed with SigmaStat 12 software using analysis of variance (ANOVA, $P<0.05$) and Duncan's test. All experiments were carried out at least two times; in each treatment, at least 10 samples were measured.

Results and discussion

Nitrosative status of non-stressed Arabidopsis lines

Compared to the WT, *vtc2-3* line – in agreement with the literature (Conklin et al. 2000; Conklin 2001) – contained lower, 52% of the wild-type total AsA. Interestingly, the NO overproducer *gsnor1-3* had extremely high AsA level (almost twice as much as the WT), compared to the other lines (Fig. 1A). In this mutant, the majority of the glutathione pool is S-nitrosylated and de-nitrosylation of the GSNO is decreased because of the lower GSNOR activity (Feechan et al. 2005). In the absence of reduced glutathione, the ascorbate-glutathione cycle cannot work properly, which may lead to *de novo* AsA biosynthesis (Colville and Smirnoff 2008). It must be mentioned that there was no statistically significant difference between the oxidised AsA content of the different lines.

In order to check the NO or $ONOO^-$ dependence of the applied fluorophores, control experiments were conducted. The inducing effect of NO-donor (SNP) and the decreasing effect of NO scavenger (cPTIO) on DAQ fluorescence (Fig. 2A) together suggest that DAQ fluorescence detects NO in *Arabidopsis* tissues. Further results indicate (Fig. 2B) that DHR detects $ONOO^-$ but not H_2O_2.

Gsnor1-3 root tips showed 76% higher NO level those of in the WT (Fig. 1B), possibly because of the low GSNOR activity and the consequently high GSNO content serving as NO source or reservoir (Lindermayr et al. 2005). Additionally, the NO and $ONOO^-$ levels in the *vtc2-3* root tips proved to be significantly elevated compared to the wild-type (Fig. 1B and 1C).

Endogenous AsA content of *nia1nia2* line was similar

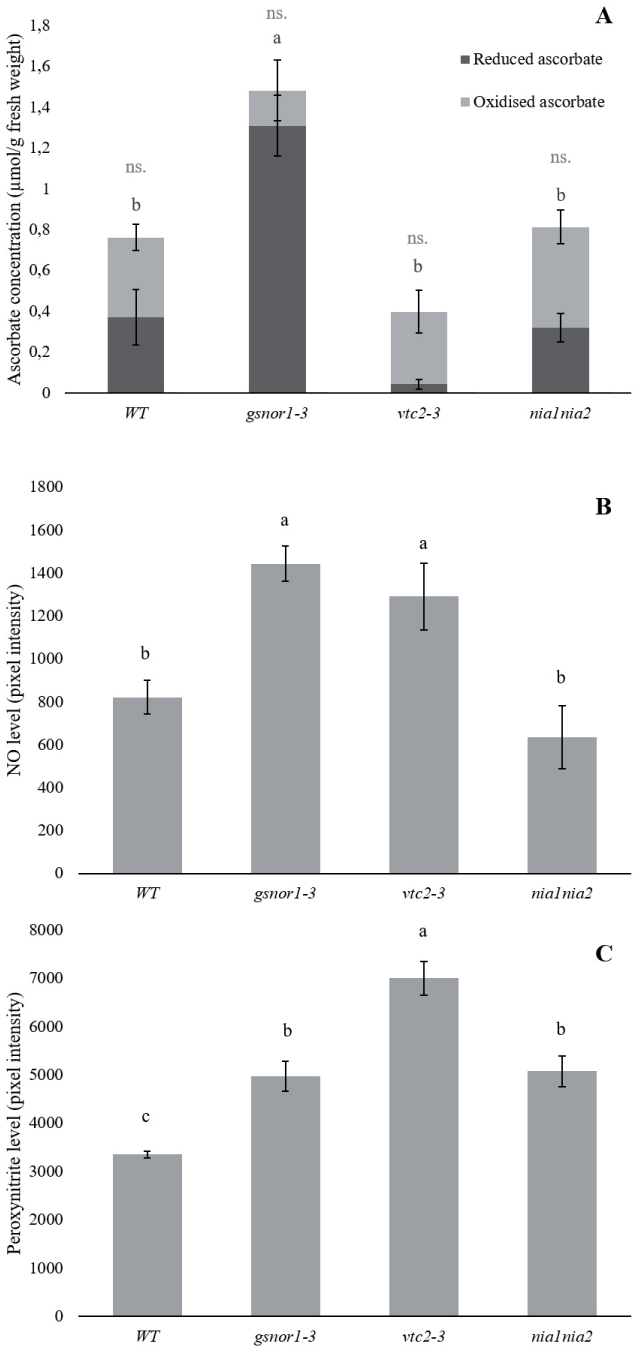

Figure 1. AsA (**A**), NO (**B**) and $ONOO^-$ (**C**) levels in 14-days-old WT and mutant *Arabidopsis* lines under control conditions. The lack of significance (n.s.) or the different letters indicate significant differences according to Duncan-test (n = 10, P≤0.05).

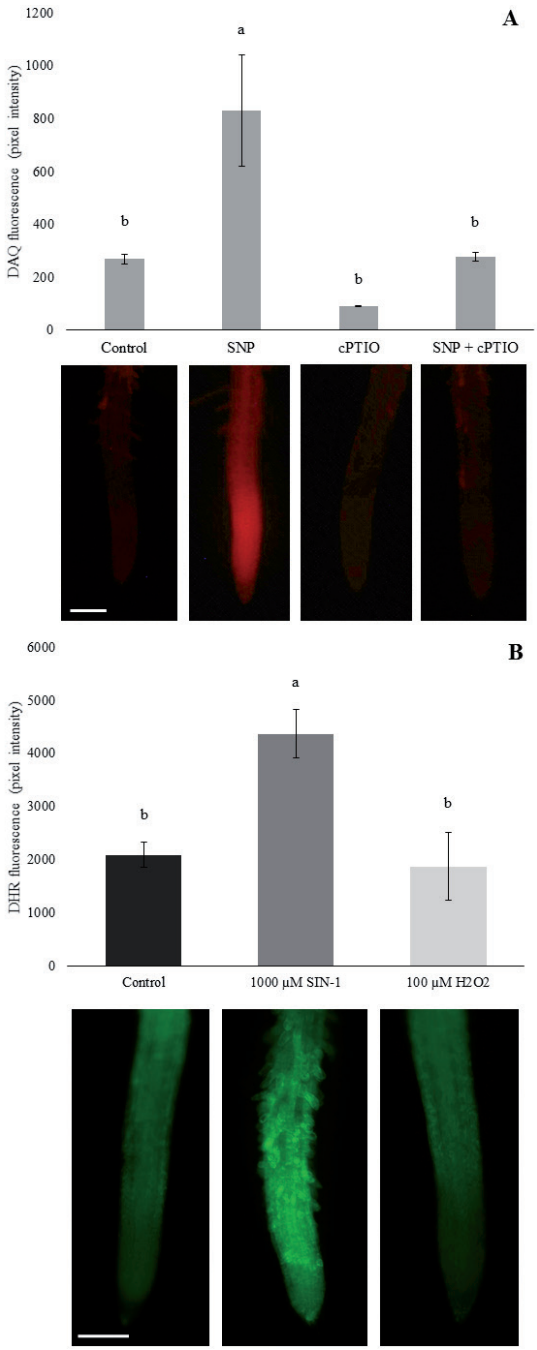

Figure 2. Fluorescent intensities and representative pictures of DAQ-stained *Arabidopsis* roots pre-treated with NO donor and/or scavenger (A), and DHR-stained *Arabidopsis* roots after peroxynitrite donor or H_2O_2 treatment (B). NO donor SNP (200 µM) and scavenger cPTIO (800 µM) were applied for one hour at 150 µmol m^{-2} s^{-1} light intensity; peroxynitrite donor SIN-1 was applied for 1 h (1000 µM) and H_2O_2 for 30 min (100 µM). Different letters indicate significant differences according to Duncan-test (n = 10, P≤0.05). Bar = 1 mm.

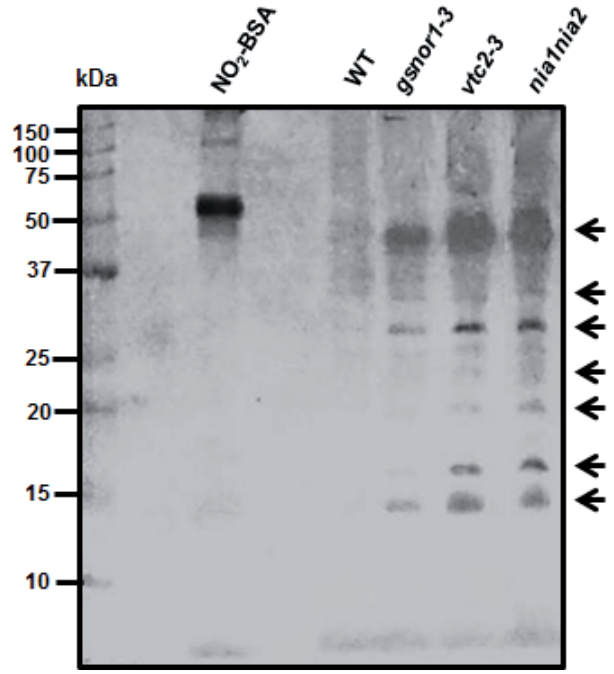

Figure 3. Representative immunoblot showing protein tyrosine nitration in 14-days-old WT and mutant *Arabidopsis* plants under control conditions. As a positive control nitrated bovine serum albumin (NO_2-BSA) was used. Arrows indicate nitrated protein lanes.

to the WT (Fig. 1A) and this line – in agreement with previous results (Pető et al. 2011) - showed lower NO level (77%) in its root tips relative to the WT (Fig. 1B). This is most likely caused by the lower activity of NR (Wilkinson and Crawford 1993), the main NO source in the roots (Chamizo-Ampudia et al. 2017). Compared to the WT, in *nia1nia2*, significantly elevated $ONOO^-$ levels were detected (Fig. 1C), which might be the result of the reaction between NO and superoxide anion. This may be supported by the previously published high superoxide radical level in *nia1nia2* roots (Pető et al. 2011).

The detectable PTN, even during control circumstances, is in accordance with previously published results (Chaki et al. 2015; Tanou et al. 2012) indicating the occurrence of physiological nitroproteome in unstressed plants. Moreover, PTN was intensified in all mutant lines compared to the WT which suggests that a bigger proportion of the proteome suffers nitration due to mutations. Interestingly, the pattern of nitration was the same in all plant lines; raising the possibility that similar proteins might become nitrated. Consequently, results show that different mutations affected the frequency of PTN, but it did not influence its pattern.

In case of *gsnor1-3,* high NO, $ONOO^-$ and AsA contents were accompanied by slightly intensified PTN compared to the WT. In contrast, the proteome of *vtc2-3* showing relatively low AsA level, but notably elevated NO and $ONOO^-$ content proved to be intensively tyrosine ni-

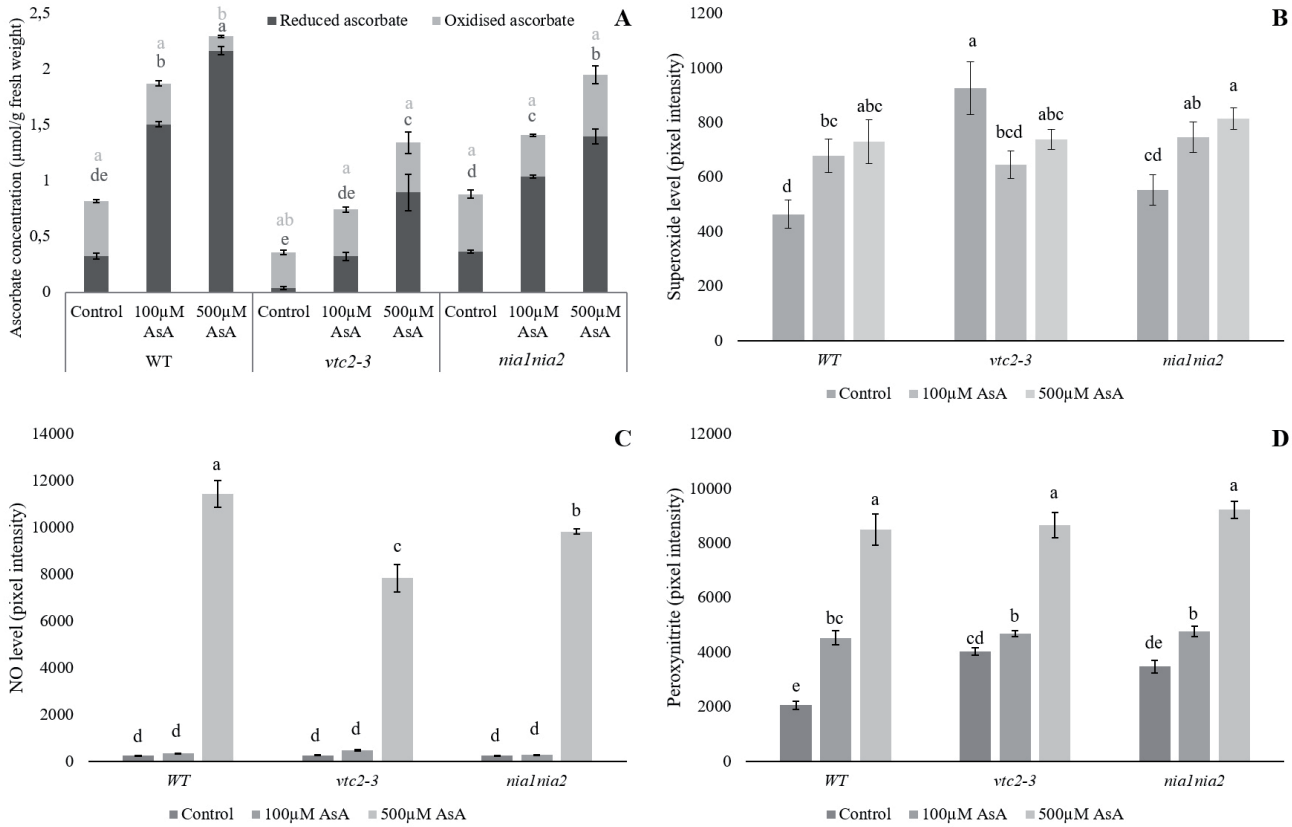

Figure 4. AsA (A), NO (B) $O_2^{\bullet-}$ (C) and $ONOO^-$ (D) levels in 14 days-old WT and mutant *Arabidopsis* lines after two weeks of exogenous AsA treatment. Different letters indicate significant differences according to Duncan-test (n = 10, P≤0.05).

trated. In NO underproducer *nia1nia2*, intensified PTN was detected, while $ONOO^-$ levels were enhanced and AsA content was WT-like. These comparisons point out that physiological protein tyrosine nitration has no tight correlation with endogenous NO content of the plant tissues. At the same time, PTN showed a positive correlation with $ONOO^-$ levels suggesting that the rate of protein nitration is associated with the tissue level of $ONOO^-$ being the source molecule of direct nitrating agents ($NO_2^{\bullet}$ and $CO_3^{\bullet-}$; Souza et al. 2008). Moreover, we found no clear relationship between endogenous AsA levels and physiological protein tyrosine nitration in *Arabidopsis* (Fig. 3).

Exogenous AsA induces PTN

Our further experiments with NO underproducer *nia1nia2* and AsA deficient *vtc2-3* lines intended to answer the question whether exogenous AsA could revert the increased PTN of these plants or AsA rather exerts pro-nitrant effect similarly to animal systems.

Significant differences were observed between the AsA accumulation properties of the *Arabidopsis* lines (Fig. 4A). Wild-type plants accumulated the most AsA in absolute value, reaching 2.29 µmol total ascorbate per one-gram fresh weight, and most of the AsA was present in the reduced form. In *vtc2-3,* 500 µM AsA treatment resulted in almost 4-fold increase in total AsA content, which is significantly larger relative increase than in the WT (3-fold). Interestingly, the AsA uptake did not decrease the quantity of the oxidised form in this case. The relative increase of AsA values in *nia1nia* was similar to WT; however, in absolute values it accumulated less AsA (Fig. 4A).

The $O_2^{\bullet-}$ content increased significantly in WT and *nia1nia2* lines as the effect of both 100 and 500 µM AsA treatment, while in *vtc2-3,* 100 µM AsA significantly decreased $O_2^{\bullet-}$ level (Fig. 4B). In *vtc2-3*, the reduced endogenous AsA content resulted in higher $O_2^{\bullet-}$ level, which might be reverted by external AsA supplementation. Despite the AsA-induced $O_2^{\bullet-}$ accumulation, there was no significant increase in lipid peroxidation (data not shown), suggesting that the externally applied AsA at these concentrations did not cause remarkable oxidative stress. In the work of Qian et al. (2014), exogenous AsA exerted pro-oxidant effect on *Arabidopsis* seedlings, although the concentrations were remarkably higher (2 mM or 8 mM) than in our experiments.

The NO content of the root tips increased significantly as the effect of the highest applied concentration, where we detected a sharp increase in NO levels in all three lines (Fig. 4C). Exogenously applied AsA induced NO accumulation also in the nitrate reductase-deficient *nia1nia2* mutant indicating that this enzyme is not involved in NO biosynthesis triggered by AsA. Rather non-enzymatic mechanisms may contribute to NO accumulation like the AsA-regulated reduction of nitrite at acidic pH (Crawford 2006) and/or the AsA-induced decomposition of GSNO-reservoirs (Kashiba-Iwatsuki et al. 1996).

The $ONOO^-$ levels of the root tips were significantly increased by exogenous AsA as well (Fig. 4D). In absolute values, all lines accumulated similar amount of $ONOO^-$ after 500 μM AsA treatment; however, in terms of relative accumulation the plant lines differed. In WT, 100 and 500 μM AsA caused 2- and 4-fold increase respectively, while in *vtc2-3,* we measured only 1.1- and 2-fold; in *nia1nia2* 1.3- and 2.5-fold increase in $ONOO^-$ levels compared to control. It should be noted, that unlike NO, $ONOO^-$ content was significantly increased by 100 μM AsA treatment as well; and in case of *vtc2-3* $ONOO^-$ level in control root tips were significantly higher than in WT. These indicate that exogenous AsA induces NO and $ONOO^-$ (representing RNS) burst in *Arabidopsis* root tips.

Then it is not surprising that 100 and 500 μM AsA significantly increased PTN in all three examined *Arabidopsis* lines. The degree of nitration was the highest in WT, but it increased remarkably also in the mutant lines (Fig. 5).

Unlike in the control experiment, the differences in the intensity of the nitration showed correlation with the NO levels, but not with the $ONOO^-$ content in case of 500 μM AsA treatment. Moreover, the most intense PTN was accompanied by the highest NO level in WT. The similar PTN levels in 100 μM AsA-treated plants seem to be connected with NO levels, as well as with $ONOO^-$ contents. Furthermore, the pattern of nitration changed compared to the control experiments, however the different AsA concentrations did not affect PTN pattern.

Exogenous AsA did not ameliorate nitrosative modification of *Arabidopsis* proteome, but it exerted a remarkable pro-nitrant effect. Moreover, the exogenous AsA-induced PTN seems to be more associated with NO level than with that of $ONOO^-$.

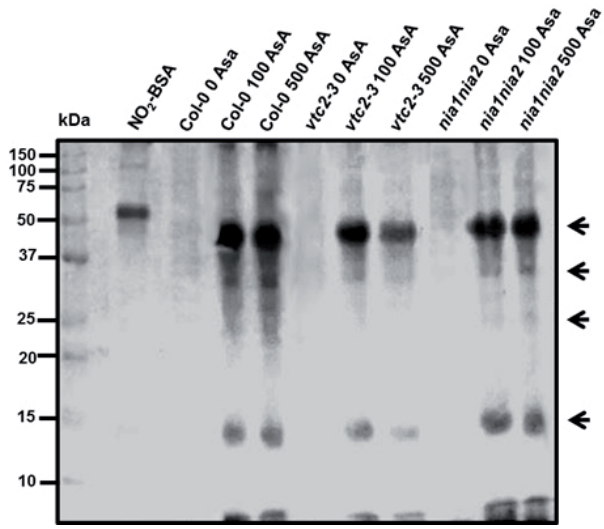

Figure 5. Representative immunoblot showing protein tyrosine nitration in WT and mutant *Arabidopsis* plants after two weeks of exogenous AsA treatment (100 or 500 μM). As a positive control nitrated bovine serum albumin (NO_2-BSA) was used. Arrows indicate nitrated protein lanes.

Conclusions

According to our knowledge, this is the first study investigating PTN in RNS/AsA metabolism mutant *Arabidopsis* under control conditions and during AsA supplementation. Data clearly show that physiological PTN in non-stressed plants is associated with endogenous peroxynitrite but not with NO levels. Furthermore, there is no correlation between the size of the endogenous AsA pool and the size of the physiological nitroproteome in *Arabidopsis*.

Applied together with abiotic stressors, AsA acts as an antioxidant (Athar et al. 2009; Chao and Kao 2010), however, its effect on healthy, non-stressed plants has been poorly studied. The limited amount of data describes the pro-oxidant and growth-reducing effect of exogenous AsA (Tyburski et al. 2012; Qian et al. 2014). In the background of the pro-oxidant effect of AsA, Qian et al. (2014) discovered the downregulation of antioxidant enzymes. This downregulation however can also be caused by protein tyrosine nitration processes, described in the present study. Thus – as a feedback loop – the failure in the antioxidant system might increase ROS accumulation, leading to the further intensification of PTN. Our results support that exogenous AsA at the applied concentrations acts as a stressor, causing RNS burst and subsequent PTN, thus it has pro-nitrant property. Interestingly, in this AsA-induced stress-situation, NO seems to be primarily connected to the development of PTN. Exogenous ascorbic acid as a pro-nitrant has been known in humans and animals for a while (Bouayed and Bohn 2010), but this is the first plant study to prove the pro-nitrant effect of this originally antioxidant molecule applied exogenously; however further research is needed to clarify the exact mechanism behind this phenomenon.

Acknowledgements

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ARTICLE

Contemporary interspecific hybridization between *Dracocephalum kotschyi* and *Dracocephalum oligadenium* (Lamiaceae): Evidence from morphological, anatomical and molecular data

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ABSTRACT *Dracocephalum* is the second largest genus in the family Lamiaceae with about 186 species. These species are native in temperate regions of the Northern Hemisphere and occur in the territory of the extra-tropical Asia and Europe. Eight *Dracocephalum* species reported in Iran; these are mainly growing in the northern and central parts of the country belonging to the Irano-Turanian phytogeographical region. *Dracocephalum kotschyi* is an important medicinal plant .in the country. At the same time, taxonomic position of *Dracocephalum oligadenium* is a challenging issue. In this work morphological, anatomical and Inter Simple Sequence Repeat (ISSR) markers were used to identify these species in Iran. MDS plot based on morphological and anatomical characters, furthermore, PCoA and MST plot based on ISSR data of species revealed hybridization between *D. oligadenium* and *D. kotschyi*.
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Introduction

The genus *Dracocephalum* L. also named dragonhead (Lamiaceae) contains about 186 species (Budantsev 1987, 1993; Kadereit 2004). *Dracocephalum* species are mostly perennial, rarely annual herbs growing in alpine and semi-dry regions. They grow mainly in temperate Asia, with a few species occurring in Europe and only one in North America (Brach and Song 2006). These species have medicinal values and are used in anti-hyperlipidemic, analgesic, antimicrobial, antioxidant, anticancer treatments (Sajjadi et al. 1998; Jahaniani et al. 2005; Sonboli et al. 2008; Zeng et al. 2010).

Budantsev (1987) divided *Dracocephalum* genus into three subgenera namely, *Dracocephalum* L., *Fedtschenkiella* (Kudr.) Schischk and *Ruyschiana* (Mill.) Briq. The subgenus *Dracocephalum* includes seven sections, the members of which have glabrous anthers and stamens.

Eight *Dracocephalum* species were reported in Flora Iranica (Rechinger 1982), while Jamzad (2012) reported 10 species in Flora of Iran. Five of these species are endemic to Iran.

The occurrence of *D. oligadenium* Bornm. & Gauba in Iran is in dispute. Esfandiari (1985) reported the occurrence of both *D. kotschyi* Boiss. and *D. oligadenium* in the country, while Jamzad (2012) considered these two as synonyms and reported only the occurrence of *D. kotschyi*.

During our extensive field collection of *Dracocephalum* species in Iran, based on morphological features we encountered the presence of both *Dracocephalum* species. Therefore, the present study has been performed to differentiate these two presumed species by multiple approaches using morphological, anatomical and molecular data. Moreover, we found plants with intermediate morphological characters. Therefore, we also tried to reveal the hybrid nature of these plants by using multiple data sets.

Materials and methods

Plant materials

Plant materials (70 plant specimens) were collected from 7 geographical populations and used for morphological, anatomical, and ISSR molecular studies (Table 1). The voucher specimens are deposited in Herbarium of Shahid Beheshti University (HSBU; Tehran, Iran).

Table 1. Geographical locations of the studied populations.

No	Province	Locality	Number of samples	Altitude (m)	Longitude	Latitude
1	Mazandran	Rineh	10	2026	35 52	52 10
2	Tehran	Fasham	10	2217	35 57	21 33
3	Qazvin	Evan	10	1796	36 29	50 26
4	Mazandran	Noor	10	2043	36 12	51 48
5	Mazandran	Namarestagh	10	2370	36 03	52 03
6	Qazvin	Niroogah	10	1318	36 17	50 01
7	Gilan	Roodbar	10	1473	36 48	49 22

Morphological and anatomical studies

Altogether, 20 morphological characters and 26 anatomical characters were studied in collected plants (Table 2 and 3).

For anatomical studies, embedded materials were prepared as follows: the adult plants samples (leaves and stems) were excised and immediately fixed for 48 to 72 h in a mixture of formalin:acetic acid:ethanol (90%) of 5%:5%:50%, respectively, than stored at 4 °C until sectioning. Samples were dehydrated in a graded ethanol series and embedded. After preparation of free transverse hand sections of the lamina and stem, samples were washed with distilled water and placed in 5% sodium hypochlorite solution for 20 min for clearing then gently rinsed with distilled water. The sections were stained with aqueous solution of methylene blue (1%) and carmine and mounted on the slides using Canada balsam (Jensen 1962). Thin cut sections were studied under a microscope fitted with digital camera. Anatomical characters of stem and leaf were summarized in Table 3.

Table 2. Morphological characteristics in the studied populations.

No.	Characters
1	Habitat form
2	Margin of stem leaves
3	Plant height (cm)
4	Length of basal leaf (mm)
5	Width of basal leaf (mm)
6	Length of petiole in basal leaf (mm)
7	Length of stem leaf (mm)
8	Width of stem leaf (mm)
9	Length of petiole in stem leaf (mm)
10	Length of inflorescence leaf (mm)
11	Width of inflorescence leaf (mm)
12	Length of petiole in inflorescence leaf (mm)
13	Size of inflorescence leaf arista (mm)
14	Length of bracteole (mm)
15	Width of bracteole (mm)
16	Size of bract arista (mm)
17	Length of calyx (mm)
18	Length of corolla (mm)
19	Width of calyx (mm)
20	Size of tooth in calyx (mm)

Morphological and anatomical characters were first standardized (Mean = 0; Variance = 1) and used to establish Euclidean distance among pairs of taxa. For grouping of the plant specimens Multidimensional Scaling (MDS) and Principal Components Analysis (PCA) were used (Podani 2000). PAST version 2.17 (Hammer et al. 2012) was used for multivariate statistical analyses.

DNA extraction and ISSR assay

Fresh leaves were collected in each of the studied populations and dried in silica gel powder. The genomic DNA was extracted using cetyltrimethylammonium bromide (CTAB)-activated charcoal protocol (Sheidai et al. 2014). The extraction procedure was based on activated charcoal and polyvinylpyrrolidone (PVP) for binding of polyphenolics during extraction and on mild extraction and precipitation conditions promoting high-molecular weight DNA isolation without interfering contaminants.

Ten ISSR primers were used: (AGC) 5GT, (CA) 7GT, (AGC) 5GG, UBC810, (CA) 7AT, (GA) 9C, UBC807, UBC811, (GA) 9A and (GT) 7CA. These were commercialized by University of British Columbia (UBC).

Polymerase chain reactions (PCR) were performed in 25 µl volumes containing 10 mM Tris-HCl buffer (pH 8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Bioron, Germany), 0.2 µM of a single primer, 20 ng genomic DNA and 3 U of Taq DNA polymerase (Bioron, Germany).

The amplification reactions were performed in a thermal cycler (Techne, Germany) with the following program: 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 sec, 57 °C for 1 min, and 72 °C for 1 min, followed by one final extension at 72 °C for 7 min. The amplification products were visualized by running on a 2% agarose gel, followed by ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

ISSR bands obtained (results not shown) were coded as binary characters (presence = 1, absence = 0). Grouping was done by Neighbor Joining (NJ) clustering, Neighbor

Table 3. Anatomical characteristics in the studied populations.

No.	Characters
1	Thickness of epidermis in stem (µm)
2	Thickness of collenchymas in stem (µm)
3	Thickness of parenchyma in stem (µm)
4	Thickness of sclerenchyma in stem (µm)
5	Thickness of upper phloem in stem (µm)
6	Thickness of xylem in stem (µm)
7	Thickness of lower phloem in stem (µm)
8	Thickness of pith in stem (µm)
9	Length of transects in stem (µm)
10	Width of transects in stem (µm)
11	Length of simple hair in stem (µm)
12	Length of glandular hair in stem (µm)
13	Number of layers of collenchymas in stem
14	Number of layers of parenchyma in stem
15	Number of layers of xylem in stem (µm)
16	Thickness of upper epidermis in leaf (µm)
17	Thickness of collenchymas in leaf (µm)
18	Thickness of parenchyma in leaf (µm)
19	Thickness of upper phloem in leaf (µm)
20	Thickness of xylem in leaf (µm)
21	Thickness of simple hair in leaf (µm)
22	Thickness of glandular hair in leaf (µm)
23	Number of layers of collenchymas in leaf
24	Number of layers of parenchyma in leaf
25	Number of layers of xylem in leaf
26	Thickness of lower phloem in leaf (µm)

Net of ordination as well as PCA. They were performed after 100 times bootstrapping/permutations (Freeland et al. 2011; Huson and Bryant 2006).

Moreover, minimum spanning tree (MST) was performed to illustrate genetic affinity of the presumed hybrid plants. Data analyses were performed by using PAST ver. 2.17 (Hammer et al. 2012).

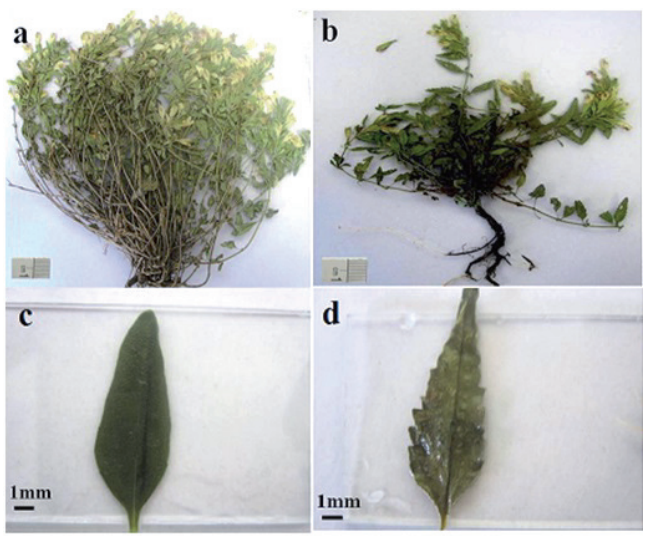

Figure 1. Habitat form and margin of stem leaves in *D. oligadenium* and *D. kotschyi*.

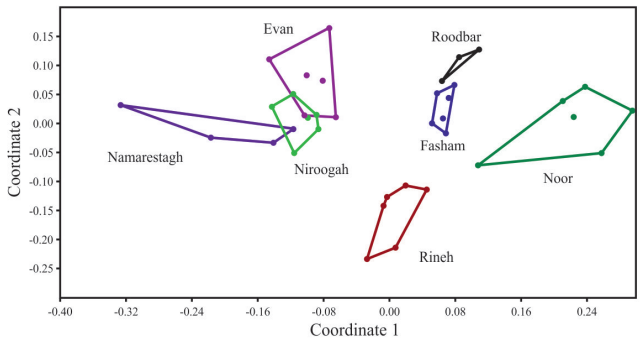

Figure 2. MDS plot of populations based on morphological characters.

Results

Morphometry

Detailed morphological investigation of the collected *D. oligadenium* (Evan, Namarestagh and Niroogah populations) and *D. kotschyi* samples (Fasham, Roodbar and Noor populations) revealed that these species mainly differ in habitat form and margin of stem leaves (Fig. 1 and Table 4). *D. oligadenium* has cartridges habitat form, while *D. kotschyi* is cartridges-cushions. Similarly, the stem leaves in the first is teethed, while they are smooth in the second one. Moreover, the plant specimens in these two species were differentiated by MDS plot based on all the studied morphological features (Fig. 2; Table 5).

We collected plants with intermediate characters in Rhine population (10 plants). They had habitat form of *D. oligadenium* and the leaf margin of *D. kotschyi*. MDS plot (Fig. 2) based on morphological characters separated *D. oligadenium* populations (Evan, Nomarestaq and Niroogah) from *D. kotschyi* populations (Fasham, Roodbar and Noor), while plants of Rineh population were placed in an intermediate position between the two species and may be considered as potential hybrids.

Table 4. Results of qualitative morphological traits in populations studies.

Species	Habitat form	Margin of stem leaves
D. oligadenium	Cartridges	Teeth of the leaves very deep
D. kotschyi	Cartridges-cushions	Teeth of the leaves not very deep

Table 5. Results of quantitative morphological traits in populations studies.

Characters	Populations						
	Rineh	Fasham	Evan	Noor	Namarestagh	Niroogah	Roodbar
Plant height (cm)	18.1	20.3	24.8	20.3	21	17.3	21
Length of basal leaf (mm)	6.2	8.3	10	9.1	8.3	7.3	10.7
Width of basal leaf (mm)	4.3	5.8	6.8	5.8	5.5	4.7	8.3
Length of petiole in basal leaf (mm)	4.1	5.7	8.8	8.2	5.8	4.7	4.3
Length of stem leaf (mm)	13	18.1	19.5	17.8	13.8	17.2	17
Width of stem leaf (mm)	6.8	8	8	8	5.8	7.4	7.5
Length of petiole in stem leaf (mm)	3	3.3	3.7	5.2	2.8	3.5	2.7
Length of inflorescence leaf (mm)	9.3	13.6	11.8	15.2	12	11.8	16
Width of inflorescence leaf (mm)	4	4.3	4.8	7	4.8	4.4	6.7
Length of petiole in inflorescence leaf (mm)	2.2	2.1	2.2	2.7	4.8	2	2
Size of inflorescence leaf arista (mm)	2.6	3	3.5	4.5	2.5	3.7	2
Length of bracteole (mm)	4.5	4.9	5.7	6.1	4.8	4.3	5
Width of bracteole (mm)	1.8	1.8	1.9	3.4	4.6	2.1	2.3
Size of bract arista (mm)	2.5	3.3	3.2	4.4	3.5	3	2
Length of calyx (mm)	16.2	14.8	16.2	14.3	13	14.7	15
Length of corolla (mm)	26.8	28.4	24.7	25.8	19.5	22.8	27.3
Width of calyx (mm)	5.3	5	4.8	4.8	4.5	5.3	5
Size of tooth in calyx (mm)	6.2	6.8	5.8	6.2	5	6.7	6

Anatomy

Details of mean of anatomical characteristics in seven studied population are provided in Table 6. PCA analysis of anatomical features revealed that the first 3 PCA components comprise about 75% of total variance. PCA revealed that anatomical characters like the thickness of collenchyma, sclerenchyma, lower and upper phloem, and xylem, as well as width of transects are the most variable anatomical characters. These characters differentiate both the species studied as well as the "hybrid population" (Fig. 3).

In this study, the stems in the cross section have a square form with pronounced angles and are covered with a one-layered epidermis. Collenchyma is single-layered among the angles but 8-11 layers of collenchyma are observed below the epidermis at the angles. Phloem and xylem were regular cylinders.

The highest thickness of parenchyma (68.81 μm), sclerenchyma (33.44 μm), lower phloem (62.4 μm) and length of glandular hair (46.51 μm) in stem was observed in Rineh population. These characters are attributes that diverge the population of Rhine from other populations.

The highest length of simple hair in stem (107.14 μm) was observed in Niroogah population, while Evan population had the highest value of thickness of epidermis (29.14 μm) and upper phloem (55.18 μm). The lowest thickness of pith (539.68 μm) and xylem (101.46 μm) in stem was observed in Niroogah.

All the leaves in the sections were bifacial (dorsiventral and amphistomatic mesophyll) type and were com-

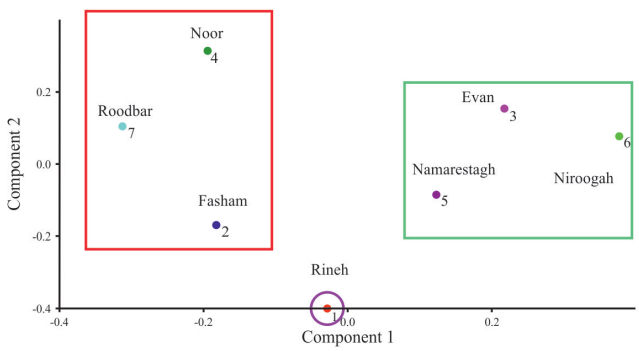

Figure 3. MDS plot of populations based on anatomical characters.

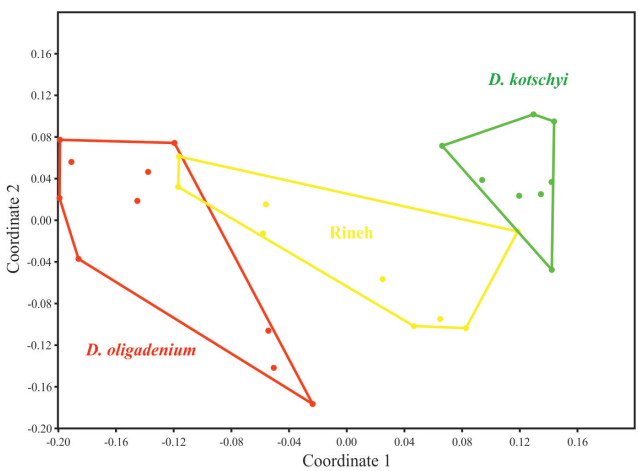

Figure 4. PCoA plot of species based on ISSR.

Table 6. Anatomical traits in the studied populations.

Characters	Populations						
	Rineh	Fasham	Evan	Noor	Namarestagh	Niroogah	Roodbar
Thickness of parenchyma in stem (µm)	68.81	50.99	60.61	49.16	63.44	61.54	43.52
Thickness of sclerenchyma in stem (µm)	33.44	15.45	30.37	20	26.34	16.66	16.66
Thickness of upper phloem in stem (µm)	39.69	28.36	55.18	35.8	38.86	46.16	30.37
Thickness of xylem in stem (µm)	146.41	152.05	157.79	165.86	162.46	101.46	214.76
Thickness of lower phloem in stem (µm)	62.4	42.81	46.86	53.68	52.68	49.16	46.86
Thickness of pith in stem (µm)	648.36	610.29	627.69	707.93	770.06	539.68	709.54
Length of transects in stem (µm)	1125.63	1111.99	1125.93	1102	1265.73	1070.68	1291.96
Width of transects in stem (µm)	1035.69	1003.26	1178.92	1154.89	1307.87	969.01	1265.76
Length of simple hair in stem (µm)	76.08	55.48	83.3	77.72	81.72	107.14	56.57
Length of glandular hair in stem (µm)	46.51	25.87	22.31	25.56	26.34	15.45	34.52
Number of layers of collenchymas in stem	8	8	11	8	11	9	10
Number of layers of parenchyma in stem	3	3	4	4	4	5	3
Number of layers of xylem in stem (µm)	12	16	20	19	20	19	18
Thickness of upper epidermis in leaf (µm)	38.33	35.25	57.14	87.41	51.11	40.41	74.51
Thickness of collenchymas in leaf (µm)	113.43	111.23	127.78	166.6	126.49	133.4	153.76
Thickness of parenchyma in leaf (µm)	234.49	140.23	143.88	193.36	247.9	225.41	179.34
Thickness of upper phloem in leaf (µm)	73.18	70.32	137.14	118.91	130.31	101.1	157.64
Thickness of xylem in leaf (µm)	185.87	157.23	208.55	199.92	206.51	159.18	165.71
Thickness of simple hair in leaf (µm)	174.07	126.32	154.39	225.12	131.55	243.31	142.97
Thickness of glandular hair in leaf (µm)	66.64	57.23	77.72	59.66	63.89	105.37	65.15
Number of layers of collenchymas in leaf	3	2	2	2	3	3	2
Number of layers of parenchyma in leaf	4	4	4	5	5	5	4
Number of layers of xylem in leaf	8	5	6	8	7	5	6
Thickness of lower phloem in leaf (µm)	174.07	126.32	154.39	225.12	131.55	243.31	142.97

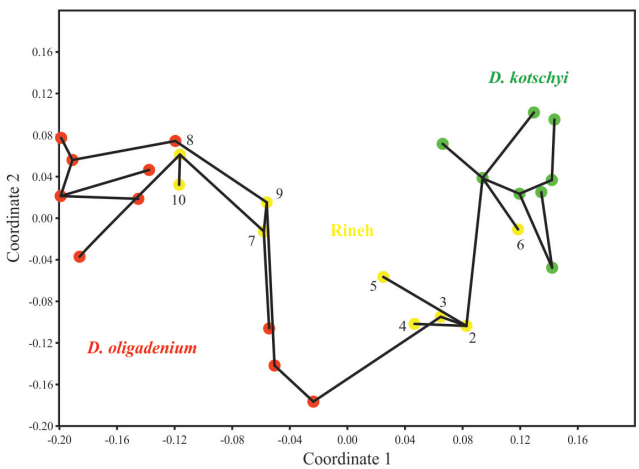

Figure 5. MST plot of species based on ISSR.

posed of one-layered epidermis. The highest thickness of the lower phloem (243.32 µm), simple (233.31 µm) and glandular (105.37 µm) hair in leaf was observed in Niroogah population and the highest thickness of the upper epidermis (87.41 µm) and collenchyma (166.6 µm) in leaf was observed in Noor population. The lowest thickness of epidermis (35.25 µm), collenchyma (111.23 µm), parenchyma (140.23 µm), xylem (157.23 µm), lower and upper phloem (70.32 and 126.32 µm, respectively), and length of glandular and simple hair (126.32 and 57.23 µm, respectively) in leaf was observed in Niroogah.

ISSR study

For ISSR studies, representatives of *D. oligadenium* and *D. kotschyi* as well as population of Rhine were analysed. PCA plot of ISSR data (Fig. 4), followed by minimum spanning tree (MST), separated *D. oligadenium* and *D. kotschyi* species from each other, while Rineh population was placed in an intermediate position. The MST plot revealed that some plants were genetically closer to *D. oligadenium* (plants 7, 8, 9 and 10 in Fig. 5); while some others were closer to *D. kotschyi* (plants 2, 3, 4, 5 and 6 in Fig. 5). This agrees with the results of the morphological and anatomical investigations.

Discussion

Species differentiation is an important taxonomic task which can be achieved through a combination of various characteristics and approaches (Sheidai et al. 2014). We could differentiate two species of *D. oligadenium* and *D. kotschyi* by using a combination of morphological, anatomical as well as molecular data. Taxonomic recognition of

these two species has been also done previously by using pollen data (Naderifar et al. 2015).

The present study also discovered hybrid plants between these two species. Natural hybridization is a widespread phenomenon in plant species and occurs in 40% of vascular plant families. The frequency of natural hybridization in plants varies among families, genera, and species pairs.

Interspecific hybridization is an important evolutionary mechanism that brings about two genomes of divergent but related species together. It can produce new genetic and phenotypic traits that can help the species ecological adaptation (Freeland et al. 2011). Interspecific hybridization occurs frequently in various plants groups but is under influence of different factors like, the genetic architecture of the species involved, the fitness of the hybrid and genotype-environment interaction (Freeland et al. 2011).

Natural interspecific hybridization occurs with high frequency in Lamiaceae family (see e.g., Gobert et al. 2002; Idowu and Oziegbe 2017; Mamadalieva et al. 2017).

The hybridization can lead to a large diversity in different characters; for example, in *Tamarix*, different species can interbreed naturally and form different hybrids with extensive range of morphological variation, depending on the degree of introgression and genetic contribution of the parental species (Ijbari et al. 2014). In case of *Helichrysum*, the hybrid plants were formed between *Helichrysum armenium* and *Helichrysum rubicundum* and showed intermediate morphological characters (Taban et al. 2015). The hybrids were formed in the hybrid zone, where the two species overlapped. The same is true in our present investigation as we collected hybrid plants in the overlapping area between *D. oligadenium* and *D. kotschyi*.

Usually, the hybrids are identified based on intermediate morphological and molecular characteristics. The same holds true for *Dracocephalum* "hybrid plants" as they were placed between the two parental taxa in MDS plot of ISSR data. Moreover, they showed intermediate anatomical features too. To our knowledge, this is the first report on the occurrence of a natural interspecific hybrid in the genus *Dracocephalum*

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ARTICLE

Morpho-meiotic study in *Mentha longifolia* from cold desert regions of Lahaul-Spiti and adjoining areas of Himachal Pradesh (India)

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ABSTRACT A morpho-meiotic study of wild *Mentha longifolia* (L.) L. (Lamiaceae) is presented from the nine populations (Kukumsari, Zero-point, Kishori, Tosh, Kasol, Key, Tiling, Mudh and Darcha) in and around the cold desert regions of Lahaul-Spiti of Himachal Pradesh. Present work is needful effort to fill the gap of morpho-meiotic (morphological and cytological) knowledge in *M. longifolia* growing in high altitude regions. Meiotic study revealed the different chromosome counts in these populations as n = 12, n = 12 + 0-3B and n = 9. Presence of B-chromosome in the species is reported for the first time from the study area and it reflects inter-population variation in five important descriptors (such as a nature of whole plant, stem, leaves, inflorescences and pollen) with 17 sub-descriptor states and occurrence of B-chromosomes. Present study reflects the existence of *M. longifolia* at diploid (2x) level based on base numbers x = 12 and x = 9.
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INTRODUCTION

Mentha longifolia (L.) L. (Lamiaceae), known well as Habek mint or Horse mint, is an aromatic perennial and tomentose herb that grows mostly in semi-shady places on moist soils (Ahmad et al. 2011; Shinwari et al. 2011). It is indigenous to Eastern Europe, Middle East, South and North Africa, Saudi Arabia and it grows wild in cold desert regions of Himachal Pradesh and in North West Himalayan belt of India. Fresh leaves and stems are mostly used for flavouring in '*salads*' and cooked foods (Facciola 1990). Like other members of the genus *Mentha* L., it is used in domestic herbal remedy, being valued especially for its antiseptic properties and beneficial effects on digestion (Karousou et al. 2007). Leaves are the chief source of essential oils, enriched in compounds like menthol, menthone, pulegone, piperitone oxides, certain monoterpenes, carvone, flavonoids, limonene and dipentene (Gulluce et al. 2007; Al-Rawashdeh 2011; Ayshath et al. 2016); and are widely used in food, beverages, flavour, cosmetics and pharmaceutical industries (Džamić et al. 2010; Bhargava 2016; Mahmoudi et al. 2016).

The species is an extremely variable taxon as large number of varieties and sub-species are reported in it (Sobti 1962) and highly variable with respect to habit, plant size, shape, denticulation and hairiness of leaves, spike length and flower colour (Aswal and Mehrotra 1994; Šarić-Kundalić et al. 2009). The information collected from the online data bases (IPCN of the w[3]TROPICOS database of Missouri Botanical Garden, Chromosome Web Watch of Japan, PhytoKaryon of University of Patras, Chromosome Counts Database, etc.), research journals and literatures (Darlington and Wylie 1955; Kumar and Subramanian 1986; Khatoon and Ali 1993; Rice et al. 2015, etc.) indicate that the species is equally variable in terms of chromosome number as 2n = 18 to 144 (Srivastava 2012; Malik et al. 2017). So far, morpho-meiotically, *M. longifolia* has not been studied from the India. Thus, present study deals with morpho-meiotic investigation of *M. longifolia* collected from the high-altitude location in and around the cold desert regions of Lahaul-Spiti of Himachal Pradesh (India). Present work is needful effort to fill the gap of morpho-meiotic (morphological and cytological) knowledge in *M. longifolia* growing in high altitude regions.

MATERIALS AND METHODS

Plant material for the present study comprised of nine populations of *M. longifolia* from Lahaul-Spiti and adjoining area of Himachal Pradesh. Plants were studied and collected in Kukumsari, Zero-point, Kishori, Tosh, Kasol,

Key, Tiling, Mudh and Darcha localities. Morphological characters were noted in the field and data were recorded at 50% flowering stage. Voucher specimens are deposited in the herbarium, Department of Botany, Punjabi University, Patiala, India (PUN). For evaluation, five important descriptors were considered, i.e. plant, stem, leaf, inflorescence and pollen. Each descriptor was further studied under different sub-descriptor states which were accessed statistically. The sub-descriptor states included were plant habit (HB), height (PH), habitat (HBT), stem hairiness (SLH), stem branching (SB), intermodal length (INL), petiole length (PL), leaf shape (LSp), dorsal (DP) and ventral pubescence (VP), presence or absence of leaf denticulation (DLP), intensity of denticulation in leaf margin (DLM), spike length (SL), flower colour (FC), flowering period (FP), pollen size (PS) and pollen fertility percentage (PF%).

For meiotic analysis the appropriate stage of young inflorescences were collected between 9.00 to 11.00 a.m. and fixed in Carnoy's fixative (6:3:1 = ethanol:chloroform: glacial acetic acid, v/v) for 24 h., after which they were transferred to 70% alcohol and stored at 4 °C. Pollen mother cells (PMCs) were obtained through standard squash technique in 1% acetocarmine. Several freshly prepared and permanent slides were carefully examined from each population to determine the chromosome number at different stages and meiotic abnormalities. Pollen stainability in glycerol: acetocarmine (1:1) was used to estimate pollen viability. For micro-photography an Eclipse 80i microscope system (Nikon) was used.

RESULTS

Presently, morpho-meiotic study was carried out in nine population of *M. longifolia* L. growing in cold desert regions of Himachal Pradesh. Morphologically, populations of *M. longifolia* were recorded with large variation in their field characters (sub-descriptor states) and clearly revealed the existence of four morphotypes viz. morphotype α, β, γ and δ. (Table 1) Scoring for the morphological characters, meiotic chromosome number (2*n*) and ploidy in each population is given in Table 1. Chromosome number (*2n*) records in *M. longifolia* and cytological information of genus *Mentha* L. from India and rest of the world is provided in Table 2 and Table 3.

Morphological study

Among the studied populations of four morphotypes in *M. longifolia* L., two morphotypes, i.e. α and β were erect

Table 1. Morphological characters in various morphotypes of *M. longifolia*.

Characters		Morphotype α			Morphotype β	Morphotype γ	Morphotypes δ
		Without B-chromosomes	With B-chromosomes	X_1	Without B-chromosomes		
	Populations	Kukumsari	Zero-point, Kishori	-	Tosh, Kasol	Key, Tiling, Mudh	Darcha
Plant	HB	Erect	Erect	Erect	Erect	Semi-erect	Semi-erect
	PH (cm)	60-150	70-150	60-150	40-80	35-50	45-70
	HBT	Moist & shady places	Moist & open slopes	Moist places	Moist places & road sides	Dry slopes & banks of Spiti river	Dry Slope near-Bhaga tributary
Stem	SLH	Non-hairy	Non-hairy	Non-hairy	Less hairy	Densely hairy	Densely hairy
	SB	Rarely 3 to 4 branched	Rarely 2 to 4 branched	Branched	Highly branched	Unbranched	Unbranched
	INL (cm)	4.6-7.7	4.2-8.1	5.72	3.4-5.2	4.4-8.2	4.5-7.6
Leaf	PL (cm)	1.3-3.2	1.4-2.9	1.73	1.4-2.5	0.6-0.8	1-1.8
	LSp	Lanceolate	Lanceolate	Lanceolate	Oblong	Oblong	Oblong
	DP	(+) low	(+) low	(+) low	(+) low	(+) high	(+) high
	VP	-	-	-	(+) low	-	(+) low
	DLP	+	+	+	+	+	±
	DLM	Conspicuously toothed	Conspicuously toothed	Conspicuously toothed	Moderately toothed	Moderately toothed	Wavy
Inflorescence	SL	9-18	7-15	12.11	6-14	2.5-4.5	2.5-4.1
	FC	Purple	Purple	Purple	Purple	Purple	Cream white
	FP	July-Sept.	July-Sept.	July-Sept.	June-Aug.	July-Aug	July-Aug
Pollen	PS (μm)	25.80 x 25.56	26.11 x 25.80	-	26.35 x 25.82	24.85 x 24.74	25.06 x 24.88
	PF (mean %)	100	73.89		94.25	93.04	93.33
	2n Count	24	24 + (1/2-3) B	-	24	24	18
	Ploidy	2x	2x	2x	2x	2x	2x

X_1 = congruent mean value for morphotype α.

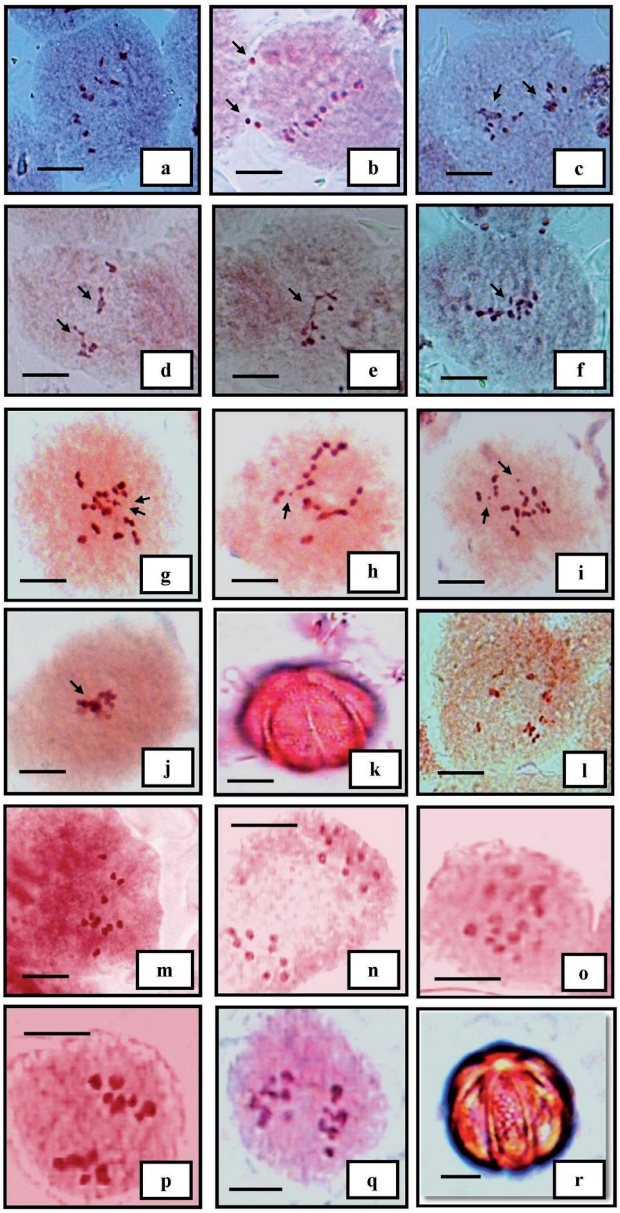

Figure 1. *Mentha longifolia* (L.) L. Morphotype α: (a) PMC showing 12_{II} at M-I; (b) PMC with 12_{II}+3B at M-I; (c-f) PMCs showing the inter-bivalent connections and chromosome stickiness at M-I; (g) PMC with a pair of 2B-chromosomes (12_{II}+2B) at M-I; (h) PMC with single B-chromosome (12_{II}+1B) at M-I; (i) PMC showing 12_{II}+2B at M-I; (j) PMC with chromosome stickiness at M-I; (k) A fertile pollen. Morphotype β: (l) PMC showing 12_{II} at M-I. Morphotype γ: (m) PMC with 12_{II} at M-I. Morphotype δ: (n-q) PMC with 9:9 chromosome distribution at A-I; (r) A fertile pollen. Scale bar = 10 µm.

while other two (γ and δ) were semi-erect in habit (HB) (Table 1). Average value of plant height (PH) varies from 60 to 150 cm in the populations of morphotype α (X_1 = 60-150 cm; PUP55036, PUP54935, PUP54929), which is followed by the plants of morphotype β (40-80 cm; Tosh = PUP55023, Kasol = PUP54937), morphotype δ (45-70 cm; Darcha = PUP55031) and morphotype γ (Key = PUP54936, Tiling = PUP55033, Mudh = PUP55004). Plants of all the morphotypes except that of morphotype δ (which were growing on dry slope near Bhaga River tributary), were reported from moist and open shady conditions.

Stem hairiness (SLH) was absent in the population of morphotype α while presents in others morphotype populations. Branching habit (SB) was observed as entire in the population of morphotype β and it is moderate in morphotype α with 2/3-4 branches per plants, while two morphotypes, i.e. γ and δ were unbranched (Table 1). Average (X_1) of variation values in inter-nodal length (INL) was less differentiable among the populations of the studied morphotypes α (X_1 = 5.72 cm; ranged b/w 4.6-7.7 & 4.2-8.1), γ (4.4-8.2 cm) and δ (4.5-7.6 cm), but it was lesser in range for the plant population of morphotype β (3.4-5.2 cm).

For the character of petiole length (PL) higher value was reported in the plants of morphotype α (X_1 = 1.73 cm), while it was lower in range for the plants of morphotype γ (0.6-0.8 cm) and intermediate in plant population of morphotype β (1.4-2.5 cm) and δ (1-1.8 cm). Leaves in all the morphotypes were oblong in shape (LSp) except in morphotype α were they were lanceolate. Leaf margin varies in denticulation (DLM) as sparse (more or less wavy in outline) in morphotype δ, moderate in morphotype β and γ to conspicuously toothed in morphotype α. For sub-descriptor state of leaf pubescence, the character of pubescence was almost present on dorsal surface (DP) of leaves of all the available morphotypes (dense and high in γ and δ morphotypes), but absent from the ventral surface (VP) in plant populations of morphotype α and γ.

Among the studied accessions of morphotypes, range of flower spike lengths (inflorescence; SL) varies as 2.5-4.1 cm, 2.5-4.5 cm, 6-14 cm, 7-15 cm and 9-18 cm in morphotype δ, γ, β, morphotype α (plants with B-chromosomes) and morphotype α (plants without B-chromosomes), respectively; whereas flower colour (FC) was either light purple (morphotype α, β and γ) or cream white (morphotype δ) in colour. Flowering period (FP) varies marginally in all the available morphotypes with the difference of one month only and it starts in month of June (morphotype β) or July (morphotype α, γ and δ) and remains in blooming condition between mid-July to August. No major differences were observed in pollen size (PS) measurement within the studied morphotypes (Table 1).

Cytological study

Morphotype α: Meiotic studies in the plants of these morphotype revealed the presence of two different chromosome numbers. Plants belonging to Kukumsari population were reported with 2n = 24 at M-I (Fig. 1a), while PMCs

in the plants of Zero-point and Kishori populations were observed to show B-chromosomes along with haploid chromosome count of n = 12 (Fig. 1b-g). The plants with and without B-chromosomes were indistinguishable in this morphotype (i.e. α). The number of B-chromosomes was observed to vary from 0 to 3 in the plants of population of Zero-point area (Fig. 1b-f), while it varied between 1 to 2 in Kishori population (Fig. 1g-i).

PMCs in the plants of Zero-point population were observed with inter-bivalent connections (37.50%) and chromosome stickiness (13.20%; Fig. 1c-f). Inter-bivalent connections (18.33%) and chromosome stickiness (7.50%) was also reported in few PMCs of the plants collected from Kishori population (Fig. 1j). Nearly 100% pollen fertility (Fig. 1k) was recorded in Kukumsari population while in plants of Zero-point and Kishori population the average pollen fertility was below 76.30%.

Morphotype β: Numerous PMCs in both the populations of this morphotype revealed the chromosome count of 2n = 24 at M-I (Fig. 1l). Meiotically the plants were normal and were recorded with high (above 90%) pollen fertility.

Morphotype γ: Meiotic analysis in the plants collected from the Key, Tiling and Mudh populations confirms the presence of 2n = 24 chromosome at M-I (Fig. 1m). Meiotic course in these plants was normal and pollens grains were cent-percent fertile (>90%).

Morphotype δ: The plants belonging to Darcha population exhibited a different meiotic count. Several PMCs at different stages of meiosis were observed with chromosome count of n = 9 (Fig. 1n). The plants of this population were perfectly normal in their meiotic course (Fig. 1o-q) and were recorded with high (>90%) pollen fertility (Fig. 1r).

DISCUSSION

Morphological variation

Morphological variation within a species is inductive of taxonomic heterogeneity and is considered as one of the fundamental factors in the process of evolutionary changes (Blinova 2012). These variations are not only helpful in establishing the connectivity of populations but also associated with the adaptability and evolution ability of taxa. It is well known fact that intraspecific morphological variations are more common in the widespread species than in the local or endemic ones (Darwin 1839; Whittakar and Fernandez-Palacios 2007). Species growing in different types of habitats also shows variations in morphological characters that are accounted for by differences in ecological conditions (Valen 1965; Stout et al. 2015). Morphological adaptations among plants to different kind of climatic and environmental conditions make them more diverse and evolved. Such plasticity in the genetic and morphological characters is more prominent among the individuals of different populations than among the members of the same population (Svensson 1983; Španiel et al. 2008). In general, morphological variations could happen due to the differences in the environmental conditions (Jones and Geber 1999; Petru et al. 2006; Stout et al. 2015), geographical boundaries (White 1971), selection and genetic drift (Abdelkrim 2005; Stuessy et al. 2006).

Variations of morphological characters among different members (i.e. species) of genus *Mentha* L. are quite common. In the presently studied species, intraspecific morphological variation was reported in different cytotypes of *M. longifolia* L. (n = 9, 12). Intraspecific morphological variation in the populations of *M. longifolia* (Table 1) might be attributed to the variation in chromosome numbers (n = 9, 12) and abnormal meiotic course, as it has been reported earlier in large number of flowering plants, e.g., *Centaurea phrygia* (Koutecký 2007), *Centaurea stoebe* (Španiel et al. 2008), *Ranunculus parnassifolius* (Cires et al. 2009), *Agrimonia* sp., *Geranium wallichianum*, *Ranunculus hirtellus* and *Vicia rigidula* (Kaur and Singhal 2010a). The presence or absence of B-chromosomes did not affect the morphological characteristics as the plants of *M. longifolia* with or without B-chromosome are indistinguishable in most of their descriptor states.

Morphological diversity among the species of genus *Mentha* is great. Due to high polymorphism, the number of species in the genus has been a matter of speculation for many years. Several features have been used in the past to examine the diversity of species using morphological (Malinvaud 1880) and cytological (Harley and Brighton 1977; Singh and Sharma 1986) aspects. Natural interspecific hybridization occurs with high frequency in *Mentha* species which may also led to morphological variation within the species. For example, cytological studies in *M. spicata* (= *M. lavaegata*) shows two cytotypes with 2n = 36 (x = 9) and 48 (x = 12) chromosomes, which differ only (except molecular) on the basis two aspects, i.e. presence or absence of non-secreting trichomes and chemical data (Gobert et al. 2002). Based on cytological and karyological data Harley and Brighton (1977) suggested the *M. spicata* (n = 24) as a hybrid of *M. suaveolens* (n = 12) and *M. longifolia* (n = 12).

Several varieties have been proposed by different authors in *M. longifolia* based on morphological and/or cytological differences, e.g., *M. spicata* var. *longifolia*, *M. sylvestris* var. *royleana* and *M. longifolia* var. *royleana*, *M. longifolia* ssp. *capensis*, *M. longifolia* spp. *longifolia*, etc. (Mukerjee 1940; Harley and Brighton 1977; Raizada and Saxena 1978; Chambers and Hummer 1994; Tarimcilar and Kaynak 2004). But currently most of these varieties

Table 2. Chromosome number (2n) count in *M. longifolia* (L.) L. from India and rest of the world (outside India).

***Mentha longifolia* (L.) L.**
India:
2n = 18: [(a)] Sobti 1962; Bala & Gupta 2012.
2n = 24: [(a,g,i)] Sobti 1965, [(a,g,i)] Sobti 1971a,b; Gohil et al. 1981; [(d)]Saggoo 1983; Bhat et al. 2002; Kaur & Singhal 2010b; [(p)] Malik et al. 2017.
2n = 27: [(a)] Sobti 1962.
2n = 36: [(i)] Arora 1960; [(a)] Sobti 1965.
2n = 48: [(a,f)] Sobti 1965.
Outside India:
2n = 18: [(d)] Schurhoff 1927, [(a,d,i)] Schurhoff 1929; [(a)] Lietz 1930; [(a,d)] Tischler 1934; [(d)] Rohweder 1937; [(a,i)] Heimans 1938; [(k)] Tarimcilar & Kaynak 2004.
2n = 24: [(a,d,i)] Ruttle 1931; [(i)] Junell 1934; [(h)] Nagao 1941; [(a,i)] Junell 1942; [(a,i)] Suzuka & Koriba 1949; Tsuda 1954; [(a,i)] Morton 1956; [(a,g,i)] Murray 1958, [(a,i)] Murray 1960; [(i)] Gadella & Kliphuis 1963; [(a,i)] Ouwenneel 1968; [(a)] Sacco & Silvano 1968; [(b)] Májovský 1970; *[(a)] Markowa & Iwanowa 1972; [(o)] Löve & Kjellqvist 1974; Fedorov 1969; [(a)] Harley & Brighton 1977; [(a,o)] Uhrikova 1978; [(b)] Májovský 1978; [(a)] Löve & Löve 1982; Fernandes & Leitao 1984; [(m)] Reitmann 1984; [(i)] Queirós 1985. Dmitrieva & Parfenov 1985; [(i)] Rosúa & Navarro 1986; Pogan et al. 1986; Parfenov & Dmitrieva 1988; [(c)] Štěpánek 1993; Khatoon & Ali 1993; [(a,i,j,k)] Chambers & Hummer 1994; Dobeš & Vitek 2000; [(l,k)] Tarimcilar & Kaynak 2004; Lawrence 2007, Al-Rawashdeh 2011.
2n = 36: [(a)] Morton 1956; [(a)] Baquar and Reese 1965; [(a)] Zhukova 1967; [(i)] Ouwenneel 1968; [(i)] Dahlgren et al. 1971; [(k)] Tarimcilar & Kaynak 2004.
2n = 48: [(a)] Nagao 1941; [(e)] Suzuka & Koriba 1949; [(a)] Pólya 1950; [(a)] Morton 1956; [(a)] Sobti 1965; [(a)] Sacco & Silvano 1968; [(d,k)] Podlech & Dieterle 1969; [(a)] Harley & Brighton 1977; Khatoon & Ali 1993; [(k)] Chambers & Hummer 1994; Murin 1997; Dobeš & Vitek 2000; [(k)] Tarimcilar & Kaynak 2004.
2n = 54: [(i)] Schiirhoff 1929; [(i)] Delay 1947a,b; Darlington & Wylie 1955; [(i)] Pogan et al. 1986.
2n = 96: Shimizu et al. 1967; [(n)] Lövkvist & Hultgard 1999.

*Note: Chromosome counts in many publications were reported with synonyms of *Mentha longifolia* (L.) L.

Synonyms:

[a] *M. longifolia* (L.) Hunds.; [b] *M. longifolia* (L.) Nath.; [c] *M. longifolia* (L.) L. subsp. *longifolia*; [d] *M. sylvestris* Linn.; [e] *M. longifolia* (*sylvestris*) [f] *M. sapida* Tausch.; [g] *M. rotundifolia* L.; [h] *M. rotundifolia*; [i] *M. rotundifolia* (Linn.) Huds.; [j] *M. longifolia* subsp. *hymalaiensis* Briq. & *M. longifolia* subsp. *capensis* (Thunb.) Briq. & *M. longifolia* subsp. *polyadena* (Briquet) Briq.; [k] *M. longifolia* subsp. *longifolia*; [l] *M. longifolia* var. *typhoides*; [m] *M. longifolia* subsp. x *M. suaveolens*; [n] *M. aquatica* var. *aquatic*; [o] *M. longifolia*; [p] *M. longifolia* L.

are treated as *sens. lat.* under same synonym of *M. longifolia* (L.) L. to cover the large range of variations (Aswal and Mehrotra 1994). From the present study area variation in hairiness of stem; size, shape and hairiness of leaves; spikes and flowers were also reported by Aswal and Mehrotra (1994). In the present case intensive screening of populations of the species from Lahaul-Spiti area revealed four morphotypes (α, β, γ and δ) occupying different locations. It seems that the phenotype of the population is greatly influenced by altitudinal range and topography. The populations of the species collected from dry temperate (Key, Tiling, Mudh and Darcha) areas show high tomentum (DP and VP) and almost woolly outlook while those of moist temperate locations (Kukumsari, Zero-point, Kishori, Tosh and Kasol) were less hairy.

The variants in the taxa are distributed in the different ecological and environmental conditions. The flowering period among the different populations varies marginally with the difference of one month. It indicates that the variation might be due to ecological preferences as suggested in other flowering plants (Korner 1999; Parker et al. 2003; Andi et al. 2011).

Morphological characteristics are the consequences of the effect of various ecological factors on the genotype of the species. Stebbins (1950) suggested that intraspecific variations of morphological characters are dependent upon the environmental modifications, genetic recombination and mutations.

Cytological illustration

Presence of 2n = 24 in the present taxa agrees with the previous reports from India and outside (Table 2). Whereas the count of 2n = 18 in Darcha population (morphotype *δ*) also confirms the previous reports from India and from the other parts of the world (Table 2). However, the present study is the first report about the presence of B-chromosome in this species (n = 12 + 0-3B).

Nearly all the described species of the genus are known cytologically (Table 3). The genus exhibits a wide range of chromosome numbers (2n = 10 to 144; highest in *M. piperita* L.; Lutkov et al. 1966; Singh 1995) which indicate the polybasic nature of the genus with base numbers x = 5, 6, 9, 10, 12 and x = 13. The base number x = 9 and 12 are the most common in distribution (Table 3). Morton's report (Morton 1973; Chambers and Hummer 1994) of 2n = 10 for *M. pulegium* from the Liège Botanical Garden makes the lowest base number x = 5 in the genus. The base numbers x = 7 and 8 are also suggested in the genus. But, true diploids (2n = 14, 16) based on these base numbers are not yet reported in nature. Singh (1995) suggested x = 12 as a secondary base number for the genus. While, Harley and Brighton (1977) suggested x = 12 as the ancestral (primary) base number for the *Mentha* L. This view was also supported by Bhat et al. (2002) and Lawrence (2007). However, literature confirms the polybasic nature of the genus with wide range of polyploids (100%) and variable reports based on different base numbers. Such a great variability in base number may be due to frequent interspecific hybridization, which makes it cytologically a complex genus.

In India nearly 13 *Mentha* species (including hybrids) are cytologically available. The species *M. longifolia* is already being reported with 2n = 18, 24, 27, 36 and 2n = 48 from different parts of the world (Table 2), it represent

Table 3. Cytological information on the worldwide and India basis in genus *Mentha* L.

	Species				Number of Species						
						Polyploidy					
	*Total	Cyto-logically counted	**Total cytotypes	2n chromosome counts (number of species)	Only 2x	Total	Intraspecific polyploidy (base number)	Level	%	Aneuploidy or with more than one base number	***Base number
World	20	[A]59	123	[B]10 (1), 12 (1), 18 (9), 20 (2), 24 (19), 26 (1), 27 (1), 30 (1), 32(1), 36 (14), 40 (2), 42 (3), 46 (1), 48 (11), 49 (1), 50 (1), 54 (5), 60 (3), 64(5), 66 (2), 68 (1), 72 (14), 74 (1), 78 (1), 84 (2), 90 (2), 92 (1), 96 (7), 98 (1), 105 (1), 108 (2), 120 (2), 122 (1), 132 (2), 140 (1), [C]144 (1)	-	[A]59	2 (8), 2 (9), 2 (10), 12 (12)	2x to 12x	100	7	(5), (6), **9**, 10, **12** & 13
India	2	[A]13	23	18 (1), 24 (2), 32 (1), 36 (4), 40 (1), 46 (1), 48 (4), 64 (1), 68 (1), 72 (2), 84 (1), 90 (1), 96 (1), 122 (1), [C]144 (1)	-	[A]13	[A]3 (12)	2x, 4x, 6x, 8x, 12x	-	2	8, 9, 10, **12**

***** Excluding hybrids/ varieties and sub-species of *Mentha*. ****** Cytotypes = Numbers of different chromosome counts within a same species. ******* Common ones bolded; doubtful ones in parenthesis; more frequently available ones bolded and underlined.

A: Number includes cytologically worked out wild and exotic / cultivated as well as hybrid species of the genus. The higher number of cytologically known species then the taxonomically recorded taxa is due to synonyms, illegitimate, invalid or unresolved names. (Major sources = Hooker 1885; Mukerjee 1940; Darlington & Wylie 1955; Fedorov 1969; Saggoo 1983; Kumar & Subramaniam 1986; Khatoon and Ali 1993; Aswal and Mehrotra 1994; Harley et al. 2004; Srivastava 2012; Rice et al. 2015; Index to Plant Chromosome Numbers: http://www.tropicos.org/Project/IPCN/; Chromosome Counts Database: http://ccdb.tau.ac.il/; The Plant List: http://www.theplantlist.org/; The International Plant Names Index: https://www.ipni.org/).

B: Lowest 2n = 10 (x = 5) in *M. pulegium* (Morton 1973; Chambers and Hummer 1994).

C: Highest 2n = 144 (x = 12) in *M. piperita* (Lutkov et al. 1966; Singh 1995).

the intraspecific chromosome variability at x = 9 and 12. Diploid nature of this species with x = 9 and x = 12 was supported by many workers (Schurhoff 1929; Morton 1956; Murray 1960; Harley and Brighton 1977; Lawrence 2007; Al-Rawashdeh 2011). Thus, present observation of 2n = 18 and 2n = 24 (or 2n = 24 + 1/2-3B) in *M. longifolia* are seems to be at diploid (2x) level which are based on x = 9 and x = 12.

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ARTICLE

Preservation effect of cinnamon and clove essential oil vapors on shelled walnut

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ABSTRACT Shelled walnut (*Juglans regia*) kernels are prone to rancidity during storage. In this study we examined the preservation effect of cinnamon and clove essential oil (EO) vapors compared to cold storage and vacuum packaging by measuring the hexanal content, indicating rancidity, in stored walnut kernels. Odor and taste of stored shelled walnut was investigated by sensory evaluation and by measuring residues of the main EO components in the kernels. During storage under EO vapors, cinnamaldehyde and eugenol were absorbed on the surface of walnuts in a time-dependent manner changing the odor and taste of the kernels. Clove (*Syzygium aromaticum*) EO prevented rancidity and EO treated kernels were rated as acceptable by the sensory panel while cinnamon EO treatment increased rancidity compared to the other treatments and the control samples.
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Introduction

Walnut (*Juglans regia*) has a valuable kernel rich in oils, vitamins, antioxidants and numerous other metabolites (Rao et al. 2016). The composition of walnuts can show slight differences by region and by variety, but proteins and fats account for more than 70% of the walnut kernel weight (Pereira et al. 2008; Tapia et al. 2013). According the study of Abdallah et al. (2015) the main volatile compounds of walnut flavors are pentanal (0.07-0.12%), hexanal (0.26-0.8%), nonanal (0.34-0.89%), 2-decenal 0.25-0.68%) and hexanol (0.21-1.58%).

High hexanal content together with the presence of 1-octen-3-ol, octanal, and 2-octenal can be found in rancid walnuts causing the characteristic odor and taste (Vidrih et al. 2012). During storage in air the unsaturated oils, especially linoleic acid, of the walnuts will be oxidized leading to compounds that decrease the chemical, nutritional and organoleptic properties of the nuts and reduce shelf life (Vaidya and Eun 2013). Auto-oxidation of oils is influenced by various factors such as oxygen, temperature, light and metal ions (Varga and Őrsi 2005). Different methods are known to prevent nuts from auto-oxidation: cooling (Laczay 2013) using antioxidant preservatives (Atarés et al. 2011; Varga and Őrsi 2013), modified atmosphere packaging or combined preservation methods (Mexis et al. 2009).

Essential oils (EOs) are originated from plant materials and their antimicrobial and antioxidant properties are well-known. About 300 EOs are commercially important (Van de Braak et al. 1999; Speranza and Corbo 2010) and some of them are used in food industry as flavorings and natural food preservatives (Hyldgaard et al. 2012). As plant-based preservatives EOs can enhance the shelf life of foods by protecting them from microbial contamination or oxidative degradation during storage and post-harvest processing (Prakash et al. 2014). Ruberto and Baratta (2000) tested about 100 pure EO compounds and have found that phenols possessed the highest antioxidant activity followed by some monoterpene hydrocarbons, namely, terpinolene, α- and γ-terpinene. The EOs used in our study had benzene ring containing compounds, cinnamaldehyde (CIN) and eugenol (EUG, a phenol), as main components (Bakkali et al. 2008). CIN is thought to be responsible for the excellent antimicrobial effect of cinnamon EO (Souza et al. 2013), and EUG, for clove EO (Vijayalakshmi et al. 2014). Both EOs were examined for their effect on retarding lipid oxidation of crude hazelnut and poppy seed oils (Özcan and Arslan 2011) and both showed significant antioxidant effect.

In this study, walnut kernels were stored refrigerated

at 4 °C or at ambient temperature in vacuum packages or in containers vaporized with cinnamon or clove essential oils. Signs of rancidity were detected by sensory evaluation and by measuring hexanal content during storage. Our goals included also the determination of adsorbed quantity of the two main components of the used EOs, CIN and EUG, and their effect on the sensory properties of walnut kernels.

Materials and Methods

Materials

Walnuts (variety Milotai 10) were obtained at a local market in Szeged, Hungary, in November and were shelled. Kernels were left to dry for 3 days. Damaged and crushed kernels or kernels with signs of mold contamination were discarded.

Cinnamon (*Cinnamomum zeylanicum*) and clove (*Syzygium aromaticum*) EOs were purchased from Aromax (Budapest, Hungary).

Sample preparation

150 g walnut kernels were placed in 720 ml sealed jars (3 jars/ treatment) with approximately 1.5 - 2 cm left between the cap of the jar and the walnuts. Storage was done at room temperature except cool storage at 4 °C. Types of treatments: unshelled walnuts (kept in textile bag at room temperature; control 1), kernels without any treatment (control 2), vacuum-packed kernels, and kernels in cinnamon or clove EO vapor. For EO vapor treatment sterile paper discs were fixed on the inside surface of jar caps with a drop of agar-agar (1.5%). The discs were impregnated with 12.5 or 25 mg essential oils giving the vapor concentration of 17 or 35 mg/l air in the closed jars.

Samples (ten pieces of kernels) were taken immediately and after 6, 13 and 18 weeks of storage and were placed in 50 ml sterile test tubes and stored at -20 °C until GC analysis. Before the sensory analysis, the samples were left on the table for 1 hour to attain room temperature.

Detection of cinnamaldehyde and eugenol residues in walnut kernels

The walnut samples (2 g) were powdered with liquid nitrogen, then suspended in 12 ml water and incubated at 4 °C for 2 hours. Then 100 µl of 10 mg/ml methyl-benzoate (Sigma, Hungary) in ethyl-acetate (VWR, Hungary) was added as internal standard and was vortexed for 0.5 min. The essential oils were extracted with 8 ml cold ethyl-acetate and the upper layer was collected after the centrifugation (3000 rpm, 4 °C, 10 min). The organic phase was dried with sodium-sulfate (Reanal, Hungary) and evaporated by IKA rotation evaporator (VWR, Hungary) approximately to 5 ml, which was followed under slight nitrogen stream up to 1 ml. After centrifugation (13 000 rpm, 4 °C, 10 min) samples were injected to an Agilent 6890N GC-FID system (Agilent, USA), using HP-Innowax (60 m x 0.25 mm x 0.5 µm) column (Agilent, USA). For carrier gas, helium was applied in constant pressure mode (32 psi), while the oven temperature was programmed from 110 °C to 250 °C with a rate of 15 °C/min, and held at the final temperature for 15 min. The injector temperature was 250 °C in split mode with 25:1 split ratio and FID detector temperature was also 250 °C. For the detector, the H_2 and air flow rates were 30 ml/min and 300 ml/min, respectively. The injection volume was 2 µl. For the quantitation, six-point calibration curves were applied for both essential oil components in the range of 40.0–1000.0 µg/ml and 33.4–834.2 µg/ml, where the y = 1.0517 x -0.001 and y = 0.8717 x -0.003 equations (r^2>0.999) were used with the 7.81 µg/ml / 20 µg/ml limit of detection (LOD) and 15.62 µg/ml / 40 µg/ml limit of quantification (LOQ) values in the case of CIN and EUG, respectively.

Detection of hexanal level in walnut kernels

Powdered walnut samples (1 g) were suspended in 2.5 ml water directly in 20 ml head space vials. Then the samples were measured using a Perkin Elmer 8500 GC-FID system equipped with a HS-101 head space autosampler (Perkin Elmer, USA), using a TG-WAXMS (60 m x 0.25 mm x 0.25 µm) column (VWR, Hungary). The initial temperature of the oven was 30 °C for 5 min, which was increased up to 200 °C with 20 °C/min and held for 2 min. The FID temperature was 210 °C, while the pressures of H_2 and air detector gases were 100 and 150 kPa, respectively. The HS-101 syringe and transfer line temperature were 110 °C, while the autosampler pretreatment program included 30 min sample incubation at 90 °C, 1 min pressurization time, 0.2 min injection time and 0.2 withdrawal time. For the hexanal calibration a six-level calibration curve (0.256 – 8.2 µg/ml) was used with the y=7.5297x-915.52 equation, where the LOD and LOQ were 48 ng/ml and 144 ng/ml, respectively.

Sensory evaluation

For sensory evaluation, a panel of five untrained judges was asked to evaluate odor and taste intensities of all samples. Taste and odor of walnut kernels was evaluated according to the following description: 5 – fresh walnut odor or taste; 4 – matured walnut odor or taste; 3 – foreign taste or odor, harmonizing with walnut; 2 - foreign taste or odor, undesirable; 1- rancid taste or odor. Evaluation was made immediately after beginning the treatments, and after 6, 13 and 18 weeks of storage. Panelists were served with fresh table water to cleanse the palate after

every sample. Samples were served on white plates and identified by a number. Samples were regarded acceptable at mark 2.5 or above.

RESULTS

Cinnamaldehyde and eugenol content of samples

The untreated samples contained, as expected, no CIN or EUG. It could be observed that EUG and CIN concentration increased with the amount of oil added (Fig 1 and 2).

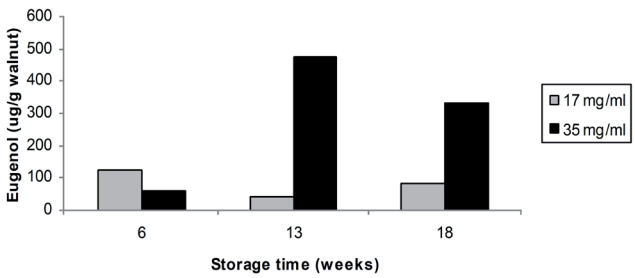

Figure 1. Eugenol content of walnut kernels treated with clove EO vapor.

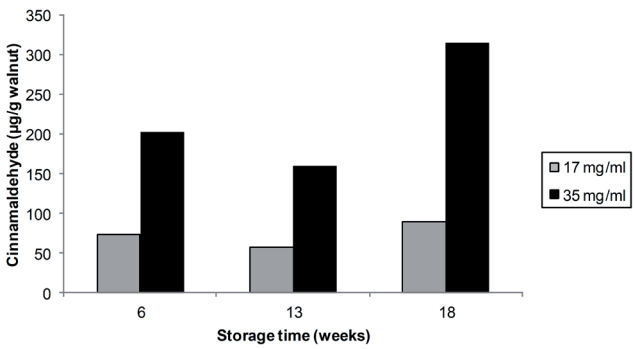

Figure 2. Cinnamaldehyde content of samples treated with cinnamon EO vapor

Hexanal (HEX) content of walnut kernels

Hexanal content of walnut samples increased in a time-dependent manner (Table 1) and reached LOQ after 18 weeks of storage. Among the non-EO treated samples, the highest amount of HEX was found in the kernels stored at room temperature, and the lowest in the ones stored in unshelled form. Using clove EO vapor, HEX content was under the LOQ during the whole storage time (Table 1). In contrast to that, cinnamon EO treated samples contained high amounts of HEX after 13 - 18 weeks of storage; higher than in any other sample (Fig. 5). It seems that in this case the EO promoted rancidity. This could be since in high concentrations cinnamon EO can work as a pro-oxidant (Bakkali et al. 2008). According to Bakkalbasi et al. (2012) and Mexis et al. (2009) the sensory limit for HEX concentration for rancid taste is 1 - 2 mg/kg walnut (1000-2000 ng/g). In kernels, treated with 35 mg/ml cinnamon EO vapor, the HEX concentration reached this value after 13 weeks and most members of the sensory panel evaluated the walnut kernels as rancid.

Sensory evaluation

At the beginning, walnut kernels were found to have a fresh, bittersweet taste and odor which during storage matured to a sweet but oilier taste. Regarding whole walnuts which were shelled just before sensory analysis, the taste at the end of the experiment was defined as undesirable although HEX content was below the sensory detection limit. This was possibly due to mold contamination vis-

Table 1. Hexanal content in the various walnut samples during storage.

Storage	Essential oil concentration (mg/l)	Storage time (weeks)	Hexanal (ng/g kernel)
Unshelled walnuts (Control 1)	0	0	-
		6	-
		13	-
		18	172.4
Kernels at room temperature (Control 2)	0	0	-
		6	-
		13	+
		18	329.4
Kernels at 4 °C	0	0	-
		6	-
		13	+
		18	325.7
Vacuum packaged kernels	0	0	-
		6	+
		13	+
		18	274.1
Kernels in clove essential oil vapor	17	0	-
		6	+
		13	+
		18	+
	35	0	-
		6	+
		13	+
		18	+
Kernels in cinnamon essential oil vapor	17	0	-
		6	+
		13	+
		18	337.0
	35	0	-
		6	+
		13	1524.7
		18	3636.4

-: below limit of detection (LOD); +: below limit of quantification (LOQ).

ible on a lot of whole walnuts and mold metabolites being responsible for the undesirable taste. Vacuum-packed and refrigerated samples were rated as having good or acceptable taste and odor throughout the experiment.

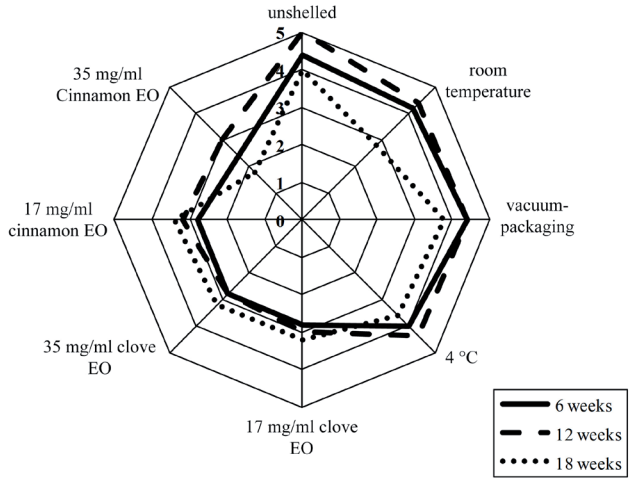

Figure 3. Average scores of the sensory evaluation (odor) of walnut kernel samples after treatment.

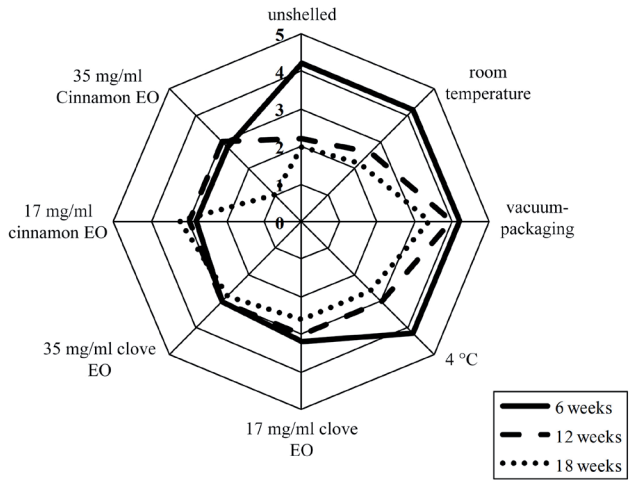

Figure 4. Average scores of the sensory evaluation (taste) of walnut kernel samples after treatment.

EO vapor treated samples had the characteristic cinnamon and clove odor but it was rated as harmonic to the walnuts' own odor. It was said to be "cake-like" odor. Taste was also rated as foreign but harmonic except samples with the highest cinnamon vapor concentration which had a rancid taste. As seen in Table 1, in this case HEX concentration reached the sensory detection limit. In general, the panelists stated that clove EO gave a pleasant taste to the walnut samples (Fig. 3 and 4).

Conclusions

Several studies demonstrated that EOs have antimicrobial, and/or antioxidant properties. An advantage of EOs is their activity in the vapor-phase: it makes them attractive for stored product protection (Krisch et al. 2013). In the recent study, cinnamon EO vapor at high concentration is not a good candidate for walnut preservation, supporting rancidity by the possible pro-oxidant effect. On the other hand, clove EO vapor can be used as potential walnut preservative at 17 mg/l concentration, giving a harmonic "cake-like" taste and odor.

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REVIEW

Application of *Saccharomyces cerevisiae* for nutritional value enhancement in agricultural plants – a review

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ABSTRACT This review focuses on yeast suspensions applied with the aim to enhance nutritional content of agricultural products. Seventy one publications were studied, and their details summarized in tables, according to the following plant groups: 1/ arable plants, 2/ vegetables, 3/ medicinal and ornamental plants. It was found that the experimental designs in these papers were inconsistent in most cases and, regardless to plant species used, the concentration of yeast extract, time of application, and repetitions of the treatment were fundamentally different, making evaluation of the methodologies difficult. However, all studies agreed in the positive impact of yeast extracts on nutritional parameters. Therefore, it is advisable to perform further studies to clarify the relationship of individual nutritional parameters to spraying dose, timing and repetition of yeast application. **Acta Biol Szeged 62(2):146-157 (2018)**

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Introduction

Although, yeast suspensions are considered as natural biostimulants in both vegetative and generative stages of plants (Ibraheim 2014), there are few in-depth studies available about the application of yeast suspensions as elicitors. According to Zlotek and Swieca (2016), there are differences in the extent of elicitation among plant species; therefore, at least species-level research is needed in this field.

With the use of scientific search engines and bibliography databases, as well as electronic libraries of universities, numerous publications related to the topic are accessible. However, these publications – especially those which connected to open field experiments and assessed by relatively simple instrumental measurements – appeared in local journals with not a real impact to the scientific field, therefore their results remained hidden. Most of the experiments reviewed in this paper were conducted in Egypt and Iraq, coordinated by agricultural universities and research stations of these countries. Soil nutrition has a critical role in successful agricultural production of these regions as sandy soil types are low in organic matter and of high percentage of degraded and reclaimed soils. Research on the use of nutrient supplementation is intensive in this region with the aim of minimizing environmental impact and production costs. Every literary source highlights the natural origin of yeast-based products as an advantage. Gawlik-Dziki and co-workers (2016) encourage the application of yeast extracts for elicitation and thus for more favorable nutritional content of the products instead of transgenic foods which have very low consumer acceptance.

Cytokinins are phytohormones having various regulatory roles in many plant processes (Kousalya et al. 2016; Macalalad et al. 2016; Parić et al. 2017). Several literature sources mention them as the key component of yeast extracts responsible for their effectiveness. As plant hormones they are used widespread in micropropagation, however, the use of the pure compounds for large scale agricultural purposes would be circumstantial and expensive. At the same time yeasts, could be good alternative sources of cytokinins and other useful constituents.

The aim of the present review was to summarize and evaluate the methodological approaches of experiments where yeast-based preparations were used for nutritional enhancement of agricultural crops.

Experimental design

Two types of basic experimental designs with focus on produce nutritional value can be distinguished, the first

investigates the effect of yeast suspensions at various concentrations, while the other compares the impact yeast suspension of with that of different materials such as natural substances (royal jelly: Fathy and Farid 1996b; methyl jasmonate: Sánchez-Sampedro et al. 2005; urea: Sarhan and Abdullah 2010; *Salix* bark extract: Gawlik-Dziki et al. 2013), plant hormones (salicylic acid: Amer 2004), vitamins (vitamin E: El-Tohamy and El-Greadly 2007; vitamin C: El-Tohamy et al. 2008; vitamin B group: Fathy and Farid 1996b; Naguib and Khalil 2002), amino acids (Hammad and Ali 2014), minerals (boron: Abou-El-Yazied and Mady 2012; zinc: Ahmed et al. 2011), fertilizers (Amino-Green: Nour and Eisa 2009; chitosan: Tartoura 2001). In the latter case, maximum two concentrations of yeast suspensions are applied, and yeast is rather a reference point, which novel products are compared to. Combinations of such materials and yeast are also assessed in most cases.

In case of open field trials, the experimental duration is generally two years in order to overcome weather extremities. Soybean (Mekki and Ahmed 2005), sugar beet (Agamy et al. 2013), and lupine (Khalil and Ismael 2010) were investigated in greenhouse pot experiments. Abbas (2013) designed his research with green bean also in greenhouse. *In vitro* experiments have also been conducted on flax cell cultures (Shams-Arkhani et al. 2005) and on broccoli sprouts (Gawlik-Dziki et al. 2013). Sánchez-Sampedro et al. (2005) used *Silybum marianum* cell cultures for assessing silymarin accumulation in relation to foliar spraying of yeast extracts. *In vitro* hairy root cultures of red sage (Yan et al. 2006) and wormwood (Putalun et al. 2007), treated with yeast extract, were analyzed for bioactive substances.

Table 1. Application of yeast solutions on arable plants.

Plant species	Variety	Application	Concentration	No. of seasons	Frequency of treatment	Reference
Beta vulgaris subsp. *vulgaris* convar. *vulgaris* var. *altissima* / Sugar beet[2]	'Hind'	greenhouse, pot, soil	50, 100 ml/pot *10^8 cfu/ml	2	When sown, repeated every third week	Agamy et al. 2013
	'Pleo'	open field, plant, foliar	10, 14 g/l	2	30, 45, 60 DAS	Neseim et al. 2014
Chenopodium quinoa / Quinoa	n.a.	open field, plant, foliar	5, 10, 15 g/l	2	45, 60 DAS	Abdallah et al. 2016
Glycine max / Soybean[1]	n.a.	greenhouse, pot, foliar	1 g/l	2	45, 60 DAS	Mekki and Ahmed 2005
	'OAC Champion'	open field, plant, foliar	1, 2, 3, 4 g/l	n.a.	R3 and R4 stages	Al-Tawaha and Al-Tawaha 2017
Linum usitatissimum / Flax	n.a.	in vitro culture	0.25, 0.8 g/l	n.a.	n.a.	Shams-Ardakani et al. 2005
Lupinus albus / Lupine	'Balady'	greenhouse, pot, foliar and/or soil	8 g/l	2	45, 59 DAP	Khalil and Ismael 2010
	'Giza-1', 'Giza-2', 'Giza-3', 'Giza-1'	open field, plant, foliar or soil	90 ml/l	2	45, 60 DAS	Mahmoud et al. 2016
Triticum aestivum / Wheat	'Bogatka', 'Mulan', 'Muszelka'	seeds, incubator	10 g/l	n.a.	4 days after germination	Gawlik-Dziki et al. 2016
	'Sakha 94'	open field and pot, plant, foliar	3, 6 g/l	2	25, 40, 55 DAS	Hammad and Ali 2014
Vicia faba / Faba bean	'Giza 2', 'Giza 3', 'Giza 843', 'Sakha 1', 'Sakha 4'	open field, plant, foliar	5, 10 g/l	2	35 DAS, 50 DAS	El-Shafey et al. 2016
	'Giza 3'	open field, plant, foliar	25, 50 ml/l	2	30, 50, 70 DAS	Mady 2009
	'Cyprus'	open field, plant, foliar	3, 6 g/l	2	35, 50 DAS	Marzauk et al. 2014
	'Super Aquadulse'	open field, plant, foliar	2.5, 5 ml/l	2	35, 50, 65 DAS	Abou El-Yazied and Mady 2012
Vigna unguiculata / Cowpea	'Creem-7'	open field, plant, foliar	25, 50 ml/l	n.a.	n.a.	Fathy and Farid 1996a
Zea mays / Maize	'TWC 352'	seeds, germination test	0.1 g/l	n.a.	Soaked for 6, 12, 18 hours	Kandil et al. 2015

The applied strains were: [1]*Candida tropicalis*; [2]*Kluyveromyces walti*, *Pachytrichospora transvaalensis*, *Saccharomycopsis cataegensis*.
DAS: days after sowing. DAP: days after planting. n.a.: not applicable/available. cfu: colony forming unit.

Plant species

Articles focusing only on vegetative growth were also excluded as the positive effect of yeast extracts on plant development seems to be obvious; the explanations in most cases refer to the cytokinin, vitamin, enzyme, and mineral content of such extracts.

With regards to arables (Table 1), the most frequent plant subgroups used were leguminous ones: faba bean, soybean, lupine, and cowpea. Leguminous plants are important vegetables in Egypt (Abdel-Hakim et al. 2012), for human consumption and as animal forage as well, being cheap sources of proteins, carbohydrates, vitamins, and minerals. El-Shafey et al. (2016) compared five faba bean varieties in an open field experiment. Maize and wheat seedlings were treated by Kandil et al. (2015), and Gawlik-Dziki et al. (2016), respectively, in *in vitro* experiments.

There are 41 articles on vegetables reviewed here (Table 2), out of which 17 apply leguminous species. The most frequent one is snap bean, followed by pea, and common bean. As previously mentioned, the importance of these plants in human consumption is high, and their cultivation improves soil mineral content due to their symbiosis with *Rhizobium* bacteria. Ten studies investigated *Solanaceae* species (potato, tomato, sweet pepper, and eggplant), six experiments dealt with *Cucurbitaceae*, while four with *Alliaceae*.

Table 2. Application of yeast solutions on vegetable species.

Plant species	Variety	Application	Concentration	No. of seasons	Frequency of treatment	Reference
Allium cepa / Onion	'Giza 20', 'Super X'	open field, plant, foliar	1, 2, 3 g/l	2	every week starting 30 DAS	Fawzy et al. 2012
	'Giza 6 Mohassan'	open field, plant, foliar	0.5, 0.75, 1 g/l	2	60, 81 DAS	Abdel-Moneim et al. 2015
Allium sativum / Garlic	'Balady'	open field, plant, foliar	2 g/l	2	30, 45, 60, 75 DAS	Shalaby and El-Ramady 2014
	'Clone sids-40'	open field, plant, foliar	2, 3, 4 g/l	2	30, 45, 60, 75 DAS	Ahmed and Farm 2015
Capsicum annuum / Sweet pepper	'California wonder'	open field, plant, foliar	1, 2, 3 g/l	2	30 DAP	Ghoname et al. 2010
	'California Wonder'	open field, plant, foliar	25, 50 ml/l	n.a.	n.a.	Fathy and Farid 1996b
Cucumis melon / Ananas melon	'Ananas'	open field, plant, foliar	50, 100 ml/l	2	25, 35, 45, 55 DAS	Adb El-Aal 2012
Cucumis sativus / Cucumber	'Safa 62'	open field, plant, foliar	2 g/l	2	n.a.	Farag 2016
	'Celerbity F1'	greenhouse, plant, foliar	1, 2, 3, 4 g/l	2	25 DAT, 32, 39, 46 DAP	Shehata et al. 2012
	'KUC-102'	open field, plant, foliar	5, 10, 15, 20 g/l	2	21 DAS	Nassef and El-Aref 2017
	'Shadi'	greenhouse, plant, foliar	6 g/l	n.a.	20, 30, 40 DAS	Sarhan et al. 2011
Cucurbita pepo / Squash	'Eskandrani'	open field, plant, foliar	0.005 g/l	2	n.a.	Abou El-Nasr et al. 2001
Cynara cardunculus var. *scolymus* / Artichoke	'Fuseau'	open field, plant, foliar	5, 7 g/l	2	50, 65, 80, 95 DAE	Hafez 2013
Ipomoea batatas / Sweet potato	'Abees'	open field, plant, foliar	5, 10 g/l	2	21, 35 DAS	El-Tohamy et al. 2015
Lactuca sativa / Lettuce	'Lymor'	open field, plant, foliar	2, 4 g/l	2	28, 42 DAP	Fawzy 2010
	n.a.	growth chamber	10, 100 g/l 1.5 ml/plant	n.a.	21, 42 DAS	Zlotek and Swieca 2016
	'Balady'[1]	open field, plant, foliar	4 ml/l 5×10^6 cfu/ml	2	30, 45 DAP	Farrag et al. 2016
Phaseolus vulgaris / Common bean	'Giza 3'	greenhouse, pot, foliar	5 g/l	1	30, 45, 60, 75 DAS	Abbas 2013
	n.a.	open field, plant, foliar	0.005 g/l	1	n.a.	Fathy and Farid 1996a
	'Bronco'	open field, plant, foliar	1, 2 g/l	2	n.a.	Amer 2004

Table 2. Continued.

Plant species	Variety	Application	Concentration	No. of seasons	Frequency of treatment	Reference
Phaseolus vulgaris / Kidney bean	'Giza 6'	open field, plant, foliar	25, 50, 100, 150 ml/l	2	28, 42 DAS	Nassar et al. 2011
Phaseolus vulgaris / Snap bean	n.a.	open field, plant, foliar	2, 4 g/l	2	n.a.	Nour and Eisa 2009
	'Bronco'	open field, plant, foliar	5, 10 g/l	2	20, 34 DAE	El-Tohamy and El-Greadly 2007
	'Poulista'	open field, plant, foliar	4, 8, 12 g/l	n.a.	30, 40, 50 DAS	Abdel-Hakim et al. 2012
	'Primel'	pot,	12 g/l	n.a.	14, 28, 42, 56 DAS	Al-Amery and Mohammed 2017
	'Pulista'	open field, plant, foliar	2, 4 g/l	2	28, 42 DAS	Fawzy et al. 2010
	'Poulista'	open field, plant, foliar	3 g/l	2	three-leaves-stage, 7, 14 days later	Byan 2014
	'Bronco'	open field, plant, foliar	25, 50 ml/l	n.a.	n.a.	Fathy and Farid 1996b
Pisum sativum / Pea	n.a.	open field, plant, foliar	100, 300 g/l	2	n.a.	El-Desuki and El-Greadly 2006
	'Balmoral'	open field,plant, foliar and soil	4 g/l	2	30, 44 DAS	Elsharkawy 2013
	'Master B'	open field, plant, foliar	30 ml/l	n.a.	n.a.	Tartoura 2001
	'Master B'[2]	open field, plant, foliar	n.a.	2	n.a.	Zaghloul et al. 2015
	'Victoria Freezer'	open field, plant, foliar	2.5, 5, 7.5 g/l	2	30, 45, 60 DAS	Ibraheim 2014
	'Gaint'	open field, plant, foliar	2, 4 g/l	2	20, 33, 48 DAS	Ali and Abd-Allah 2010
Solanum lycopersicum / Tomato	'Super Strain B'	open field, plant, foliar	15, 30 g/l	2	20, 35, 50, 65, 80 DAP	El-Desouky et al. 2011
	'Castel Rock'	open field, plant, foliar	25, 50 ml/l	n.a.	n.a.	Fathy and Farid 1996b
	'Super Strain B'	open field, plant, foliar	2, 4 g/l	2	30, 45, 60 DAP	Abou El-Yazied and Mady 2011
Solanum melongena / Eggplant	'Black Beauty'	open field, plant, foliar	5, 10 g/l	2	30, 45 DAP	El-Tohamy et al. 2008
Solanum tuberosum / Potato	'Desiree'	open field, plant, foliar	2, 4, 6, 8 g/l	n.a.	30, 40 DAP	Hussain and Khalaf 2007
	'Desiree'	open field, soil	2, 4, 6 g/l 1 l/m^2	2	5, 15, 25, 35 DAE	Sarhan and Abdullah 2010
	'Riviera'	open field, soil	4, 8 g/l	1	germination stage, tuber formation stage	Kahlel 2015
	'Valor'	open field, plant, foliar	0, 1, 2, 3, 4, 5 g/l	2	30, 44, 58 DAS	Ahmed et al. 2011
Brassica oleracea convar. *botrytis* var. *italica* / Broccoli	'Cezar'	in vitro, germination test, watering, sprouts	1, 5, 10 g/l	n.a.	2, 3, 4 DAS	Gawlik-Dziki et al. 2013

The applied strains were: [1]Sc NCAIM Y 00216; [2]local isolate; [3]Vi-cor® company. DAS: days after sowing. DAE: days after emergence. DAP: days after planting. n.a.: not applicable/available. cfu: colony forming unit.

A non-comprehensive collection of fifteen articles using yeast extracts on medicinal and ornamental plants were also included into this review (Table 3) to provide an insight into advantages of this approach in this group of plants. In case of medicinal plants, the focus was on the amount and composition of the essential oils produced. Limited number of sources was found on treatment of fruit species; therefore, this plant group has been excluded from this review.

Yeast species and extracts

The raw material used for suspensions are not well defined in most cases. The names active dry yeast, or active yeast extract, brewer's yeast, bread yeast, instant yeast are generally used. The origin of the strain is not sufficiently documented either. In contrast, Agamy et al. (2013) provided the names of the three yeast strains obtained from a personal collection of South Africa. Nassar et al. (2015) used commercial yeast powder, while Sánchez-Sampedro et al. (2005) applied the aqueous extract of crude yeast. Abou El-Yazied et al. (2011) defined an American company (Vi-COR) as the source of the yeast material. Farrag et al. (2016) shared the catalog number of the strain used (Sc. NCAIM Y 00216), provided by Sakha Agricultural Research Station, Egypt (but the number proved misleading and untraceable). Zaghloul et al. (2015) used a local isolate for pea experiments. Mekki and Ahmed (2005) incorrectly identified baker yeast with *Candida tropicalis*, an opportunistic pathogenic yeast. Several articles, such as Abou El-Yazied and Mady (2012) or Khalil and Ismael (2010) cited sources (e.g., Mahmoued 2001; Nagodawithana 1991) about the compounds of yeast extract in general; but failed to provide the analysis of the actual material used. Therefore, the comparability of these experiments is questionable due to the lack of a standardized yeast material or of any nutritional parameters as base for comparison. None of the articles refer, for instance, to USDA (2016) database, where nutrient component quantities are reported. That database also defines that both names of baker's and active dry yeast can be used. The term brewer's yeast can refer to various *Saccharomyces* strains (Kurtzmann and Robnett 2003), therefore, it is inevitable to provide detailed information about the yeast material used for ensuring the repeatability and comparability of results.

Yeast suspension/ extracts preparation

In most cases, the preparation of yeast suspensions follows the same pattern: active dry yeast is suspended in a water-sugar (1:1) solution (Ahmed et al. 2011; Byan 2014). After

Table 3. Application of yeast solutions on medicinal and ornamental plant species.

Plant species	Variety	Application	Concentration	No. of seasons	Frequency of treatment	References
Artemisia absinthium / Wormwood	n.a.	growth medium, hairy roots	0.5, 1, 2 g/l	n.a.	21 DAS	Putalun et al. 2007
Borago officinalis / Borage	n.a.	open field, plant, foliar	2, 4, 6 g/l	2	150, 180 DAS	El-Din and Hendawy 2010
Carum carvi / Caraway	n.a.	open field, plant, foliar	1, 2g/l	2	40, 60 DAS	Medani and Taha 2015
Coleus blumei / Coleus	n.a.	in vitro culture, medium	0.01, 0.025, 0.05, 0.1g/l	n.a.	8 DAS	Sahu et al. 2013
Coriandrum sativum / Coriander	n.a.	open field, plant, foliar	1, 2, 3 g/l	2	n.a.	Eid 2001
Geranium sp. / Geranium	n.a.	greenhouse, pot, foliar	2, 4, 6 g/l	2	1 and 2 week(s) before cutting	El-Lethy et al. 2011
Melissa officinalis / Lemon balm	n.a.	open field, plant, soil	5, 10, 15 g/l	2	15, 36 DAP	Rashed 2012
Nigella sativa / Black cumin	n.a.	n.a.	0.002 g/l	n.a.	n.a.	Naguib and Khalil 2002
Ocimum basilicum / Basil	n.a.	open field, plant, foliar	2, 4, 6 g/l	2	30, 44 DAS, and one month after first cut	El-Nagger et al. 2015
	n.a.	open field, plant, foliar	4 g/l (2, 8, 12)	2	49, 70 DAS	Nassar et al. 2015
Salvia miltiorrhiza / Red sage	n.a.	in vitro, hairy roots	0.05, 0.1, 0.2, 0.4 g/l	n.a.	18 days after inoculation	Yan et al. 2006
Salvia officinalis / Sage	n.a.	open field, plant, foliar	0.1, 0.2, 0.3 g/l	2	n.a.	Massoud 2006
Silybum marianum / Milk thistle	'Albiflora'	open field, plant, foliar	25, 50, 100 g/l, 1100 l/ha, 5 l/plot	2	14, 75 DAS	Saad-Allah et al. 2017
	n.a.	in vitro cell culture	0.005, 0.015, 0.025, 0.05, 0.1 g/l	n.a.	n.a.	Sánchez-Sampedro et al. 2005
Stevia rebaudiana / Stevia	'Spanti', 'China-1'	open field, plant, foliar	2, 4 g/l	2	30, 60 DAS	Salama et al. 2016

DAS: days after sowing. DAP: days after planting. n.a.: not applicable/available

that an overnight period (El-Tohamy and El-Greadly 2007; Abbas 2013), or two days (Ahmed et al. 2011) is provided for the activation and growth of yeast cells.

Most of the articles – especially those from Egypt - cite and use this method. Others, *e.g.*, Abou El-Yazied and Mady (2012), Barnett et al (1990), Nassar et al (2011), Hafez (2013), Mahmoued (2001), or Saad-Allah et al. (2017), refer to Spencer et al. (1983). In these works, dry yeast powder was activated by using 6:1 ratio of carbon and nitrogen sources. According to the authors, the highest cell number of yeasts can be achieved with this method: each ml of activated yeast culture contains about 1.2 x 10^4 yeast cells. In this work of Spencer et al. (1983) various details are given (e.g., on budding and growth rate of *S. cerevisiae* in different environments), however, no information provided about how to prepare an extract from yeasts.

There are several frequently applied methods for releasing beneficial bioconstituents from yeast cells. Bartlett et al. (1990) used a medium with glucose and casein as favorable sources of C, N and other essential elements in a suitable balance, and adjusted air supply and temperature. The culture was subjected to two cycles of freezing and thawing for disruption of yeast cells, directly before use. Tween-20 detergent is added for tested treatments in the experiment of Bartlett et al. (1990). However, in Spencer et al. (1983) no method for preparing an effective yeast extract is given.

Farrag et al. (2016) followed the method of Ono et al. (1991), where Yeast Peptone Glucose (YPG; 2% glucose, 2% peptone, and 1% yeast extract) medium was used for the propagation of yeast cells. The growth temperature was 30 °C. The liquid culture was later used for foliar treatments. Yan et al. (2006) used the carbohydrate (polysaccharide) fraction of a commercial yeast extract (Y4250; Sigma, St. Louis, MO). The suspension for foliar treatments was prepared by a two-times ethanol precipitation then dissolved in 100 ml distilled water, sterilized by autoclaving (121 °C, 20 min), and stored in a refrigerator at 4-8 °C before use. The dose was expressed by the total carbohydrate content determined by the phenol-sulfuric acid method using sucrose as a standard (Yan et al. 2006).

Zlotek and Swieca (2016) followed the method of Gawlik-Dziki et al. (2013), where instant yeast is suspended in distilled water in certain concentrations, then autoclaved, with the addition of Tween-20 as surfactant.

Targeted plant part, timing and frequency of treatments

The most common application, especially in case of open field experiments, is foliar spraying. Agamy et al. (2013) did soil inoculation with yeast suspensions in a pot experiment with sugar beet. Mahmoud et al. (2016) compared foliar and soil application of yeast on five lupine varieties. Kahlel (2015), and Sarhan and Abdullah (2010) applied soil inoculation on potato test plants. Sahu et al. (2013) applied yeast extracts in the growth media for an *in vitro* experiment on *Coleus* sp. Kandil et al. (2015) soaked maize seeds and assessed certain nutritional parameters of the radicle.

With regard to arables, treatments were applied two or three times, starting about 30 days after sowing, and repeated after 15-20 days. In case of experiments with faba bean varieties, the authors (*e.g.*, Mady 2009; Marzauk et al. 2014; El-Shafey et al. 2016) did not refer to other works regarding experimental designs, while those with lupine were consistent even though the environment was different (greenhouse/open field).

In case of vegetables, the number of treatments is often increased to four, starting generally after 30 days and keeping 10-15 days intervals. Experiments performed with garlic varieties are consistent in timing and frequency of treatments. Experiments with lettuce originating from three different research groups show some similarities in performing the treatments although the conditions were different. Publications describing experiments on various bean types do not provide all details but the development of the methodology is probable; two applications of Fathy and Farid (1996a) are gradually increased to four by Al-Amery and Mohammed (2017). Experiments done with pea, tomato, and potato show limited methodological similarities within a variety.

No coherence is seen between the applied methodology and the investigated plant part; leaf vegetables are treated twice, fruit, tuber, and bulb vegetables are sprayed 2-4 times independently from foliage size or the position of marketable plant part. Out of 71 reviewed papers, only one examined the effect of the number of treatments within the same experiment: Zlotek and Swieca (2016) used single and double spraying on lettuce cultivated in growth chamber.

Agamy et al. (2013) and Zlotek and Swieca (2016) refer to preliminary experiments for selecting the concentration to be used in the main experiment. However, most articles reviewed here do not provide detailed explanation on the selection of concentrations, volumes, or treatment frequencies used.

Sampling

For pigments analysis, leaves of the test plants were collected; depending on the media, species, and other parameters of the experiment, the time of leaf sampling was done 30-150 days after sowing (DAS). In case of leaf vegetables and medicinal plants, this time was the end of the season (maturity). Mady (2009), as well as Abou-El-Yazied and Mady (2012) collected leaf samples two times, with a 15-20 days interval. Neseim et al. (2014) did not

define an exact date, only stated that leaves were fresh. El-Tohamy et al. (2015) and Khalil and Ismael (2010) defined a vegetative development stage; the second and the fourth full developed leaves of sweet potato and lupine were collected, respectively.

The plants' marketable plants were sampled, for detailed nutritional analysis, typically when ordinary harvest would have taken place. Tubers, roots, bulbs, pods, seeds, spikes, shoots, and fruits were collected in full ripening and taken to instrumental measurements.

Results

Nutritional parameters investigated on leaves or shoots

Total sugars, total soluble solids and dry matter content was found to be increased in several studies.

Analysis of macronutrients (nitrogen, phosphorus, potassium) in leaves showed a general increase (eggplant: El-Tohamy et al. 2008; tomato, pepper, bean: Fathy and Farid 1996b; lettuce: Farrag et al. 2016), and so did calcium and magnesium (tomato, pepper, bean: Fathy and Farid 1996b). Fawzy (2010) measured lower levels of nitrates and higher amounts of Fe, Mn, and Zn when used the suspensions on lettuce. The same was found by Medani and Taha (2015) in caraway shoot samples.

Following yeast treatments, free amino acid content (sugar beet: Neseim et al. 2014; quinoa: Abdallah et al. 2016), protein content (faba bean: Mady 2009; sugar beet: Agamy et al. 2013) and carbohydrate levels of the plant leaf samples (bean: Fathy and Farid 1996b; Abbas 2013; wheat: Hammad and Ali 2014; caraway: Medani and Taha 2015) showed an increase. But in treated milk thistle seedlings, decrease of amino acids concentration was measured by Saad-Allah et al. (2017).

In the case of leaf or shoot samples, chlorophyll a and b, were measured, often together with carotenoids; and a general rise was observed in comparison with untreated plants. Neseim et al. (2014) found this effect to be non-significant, as well as Agamy et al. (2013) and Zlotek and Swieca (2016) within their given experimental designs.

Fawzy (2010) measured ascorbic acid changes after yeast treatments of lettuce and found a significant rise. Zlotek and Swieca (2016) could not find a significant difference in the case of lettuce.

Hammad and Ali (2014) investigated peroxidase and phenoloxidase activity, which were higher in the case of treated wheat leaves. Yan et al. (2006) found increased tyrosine aminotransferase and lower phenylalanine ammonia lyase activity in the case of treated red sage leaves.

Saad-Allah et al. (2017) investigated milk thistle seedlings for photosynthetic efficiency, which showed a non-significant increase.

El-Tohamy and El-Greadly (2007) observed an increase of auxins (IAA) and gibberellin (GA3) in bean shoots after yeast treatments. Abdallah et al. (2016) found an increase of IAA levels on quinoa leaves. The same was found by Abbas (2013), together with increased ABA+ activity, and by El-Shafey et al. (2016), together with increased cytokinin levels. Higher levels of cytokinins were also observed by El-Tohamy and El-Greadly (2007) on bean and by El-Tohamy et al. (2008) on eggplant. Besides this, Mady (2009) found an increasing amount of auxins and a lower level of abscisic acid in treated faba bean leaves. Higher amounts of auxin, gibberellins and cytokinins and the decrease of abscisic acid was supported by the results of Abou El-Yazied and Mady (2011) and Abou El-Yazied and Mady (2012) on tomato and on broad bean, respectively. Increasing levels of chichoric acid, ferulic acid, and caffeic acid was found by Zlotek and Swieca (2016) in lettuce leaves.

An increase of total phenols was experienced by Yan et al. (2006), Neseim et al. (2014), and Abbdallah et al. (2016) on red sage, sugar beet, and quinoa, respectively. Gawlik-Dziki et al. (2013) detected changes in the phenolic profile of treated broccoli sprouts; chlorogenic and p-hydroxybenzoic acid decreased, while p-coumaric and syringic acid increased, and flavonoids content was also elevated by yeast spraying; resulting in increased antiradical activity of the broccoli sprouts. Zlotek and Swieca (2016) found a non-significant increase of lettuce flavonoids content, together with increased DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) results.

Nutritional parameters investigated on seeds

Abdallah et al. (2013) measured carbohydrate and protein content increase of quinoa seeds treated with yeast extracts. The same was found by Khalil and Ismael (2010) in lupin, by Mady (2009), by El-Shafey et al. (2016) in faba bean, and by Hammad and Ali (2014) in wheat. The protein levels were consistently growing in two development stages of soybean (Al-Tawaha and Al-Tawaha 2017). The decrease of the total fiber content of wheat was observed by Hammad and Ali (2014).

The nitrogen, potassium and phosphorus content of seeds from arable plants seems to be increasing as the function of the treatment with yeast suspensions (Mekki and Ahmed 2005; Mady 2009; Khalil and Ismael 2010; Mahmoud et al. 2016).

In the study of Abdallah et al. (2016), the oil content of quinoa seeds rose insignificantly, but in soybean seeds, Mekki and Ahmed (2005) found a significant increase.

The alkaloid content of white lupin showed a significant decrease when treated by yeast suspensions (Khalil and Ismael 2010), while the results of Mahmoud et al.

(2016) showed a non-significant change in the same species.

DPPH and flavonoids content increase of treated quinoa seeds was insignificant (Abdallah et al. 2016). The phenolics content of wheat seeds treated by yeast and germinated for four days were increased according to Gawlik-Dziki et al. (2016). However, total phenolic content (TPC) changes were insignificant and inconsistent in the study of Mahmoud et al. (2016) on lupine.

Nutritional parameters investigated on roots, tubers and bulbs

The sucrose content of sugar beet roots increased when treated with yeast extracts (Agamy et al. 2013). Following to foliar treatments, the total soluble solids content of onion bulbs increased according to Fawzy et al. (2012), and Abdel-Moneim et al. (2015). The same was found by El-Tohamy et al. (2015) in sweet potato tubers and by Hussain and Khalaf (2007) in potato. In contrast, Kahlel (2015) found a non-significant total soluble solids (TSS) increase in the case of potato in a one-year trial.

An increase of starch percentage was observed by Ahmed et al. (2011) when yeast extracts were used on the foliage of potato.

The macronutrients as well as nitrate and nitrite content of onion bulbs showed an increase after foliar treatments (Ahmed and Farm 2015; Abdel-Moneim et al. 2015). In potatoes, level of macronutrients and Zn was elevated after yeast treatment (Ahmed et al. 2011). Fawzy et al. (2012) further found that Fe, Cu and Mn levels were also increased in the case of treated onion plants.

In treated sugar beet roots, increase in protein (Agamy et al. 2013), as well as in free amino acids and phenols (Neseim et al. 2014) was found. Increased protein level was found by Ahmed et al. (2011) in potato tubers. In onion bulbs, total carbohydrate content and volatile oil content were elevated on yeast treatments in the study of Ahmed and Farm (2015).

Nutritional parameters investigated on fruits

The foliar application of yeast extracts resulted in an increase of nitrogen, phosphorus and potassium in tomato fruits (Fathy and Farid 1996b; El-Desouky et al. 2011; Abou El-Yazied and Mady 2011). This is in agreement with the findings of Abd El-Aal (2012) in ananas melon, where calcium and magnesium levels were also elevated. Shehata et al. (2012) investigated the effect of yeast extracts on cucumber; elevation of Fe, Zn, Cu, and Mn, besides macronutrients, was recorded.

The TSS as well as total acid content (mainly responsible for taste properties of tomato) showed an increase in the study of Abou El-Yazied and Mady (2011). Besides TSS, total sugar levels were also increased in ananas melon (Abd El-Aal 2012) as well as in sweet pepper and in tomato (Fathy and Farid 1996b; Ghoname et al. 2010; El-Desouky et al. 2011). Increase of TSS was recorded by Sarhan and Abdullah (2011), Shehata et al. (2012), and by Farag (2016) in cucumber. In contrast, Nassef and El-Aref (2017) found no significant difference in TSS in the same species, but clear increase in the percentage of reducing sugars.

The protein and carbohydrate content of tomato were increased by yeast treatments according to Fathy and Farid (1996b), Abou El-Yazied and Mady (2011), and El-Desouky et al. (2011).

Application of yeast extracts positively affected also vitamin C content of tomato (Abou El-Yazied and Mady 2011) and of sweet pepper fruits (Ghoname et al. 2010).

Nutritional changes of artichoke inflorescence following yeast treatments – significantly increased total carbohydrate, inulin and Na content – was found by Hafez (2013).

Oil content of medicinal plants

All reviewed sources agree that foliar application of yeast extract has a positive impact on oil content of medicinal plants. In most cases, significant differences were measured, except e.g. in Nassar et al. (2015) in basil. The decrease of stevioside and rebaudioside-A percentage of stevia plant was reported by Salama et al. (2016). At the same time, a decrease in the number of volatile components on yeast treatment was seen by Nassar et al. (2015) in basil. Certain compounds of basil showed non-significant changes in the study of El-Nagger et al. (2015).

Discussion

Soil inoculation or foliar application?

Khalil and Ismael (2010) applied foliar and soil, as well as combined treatments on lupine plants. They found foliar application of yeast extract significantly more effective on the chlorophyll content of leaves than soil inoculation. Similarly, foliar application was more favorable than soil application in the case of nitrogen, protein, and carbohydrate content of lupine seeds. This is possible due to the hypersensitive reaction induced by yeast extracts.

Foliar and soil application of yeast suspensions on lupine plants was also investigated by Mahmoud et al. (2016) who found slight differences between the two methods in terms of measured nutritional parameters. Protein and lipid percentage, as well as TSS were higher, while alkaloids were lower in case of foliar application. These results were consistent through the dataset of three cultivars, while TPC was higher in two out of three varieties when applied on foliage.

Which is the most advantageous concentration?

In most studies, higher concentrations of yeast suspensions generally resulted in higher nutritional values, and to some extent a linear positive correlation was seen. Even the highest concentrations used in the referred studies had no deteriorative effect on the nutritional parameters of test plants. However, in some cases yeast extracts had no significant impact on the investigated characteristics, and in certain studies, non-linear correlations could be assumed. For example, El-Naggar et al. (2015) found contradictory results as to the optimal concentration regarding leaf pigments of basil leaves; in the first season, concentration of chlorophyll was the highest when 6 g/l suspensions were used, while in the next year lower concentrations gave better results. Similarly, GC-MS analysis of volatile components revealed that different concentrations were advantageous for the enhancement of each volatile. Eid (2001) found a reverse effect of yeast extract concentration on essential oil content of coriander plants; 1 g/l was more advantageous than either 2 or 3 g/l. Similarly, Sánchez-Sampedro et al. (2005) found that not the highest applied concentration was the most advantageous for enhancing silymarin content of *Silybum marianum* cell cultures.

Al-Tawaha and Al-Tawaha (2017) found that 1 g/l, the applied lowest concentration, increased crude protein levels of soybean the most, while 2 g/l enhanced fiber and oil content. Higher concentrations (3 and 4 g/l) in the same study had no such outstanding effect on these parameters. Regarding protein content, Nassar et al. (2011), Marzauk et al. (2014), and Ibraheim (2014) found similar results, i.e. not the highest applied concentrations were the most advantageous. In the study of Mady (2009) the applied lower concentration (25 g/l) was more favorable for auxin concentration, while all other measured parameters were higher when 50 g/l extract was used. The same tendency was found by Abou El-Yazied and Mady (2011) on the gibberellin content of tomato samples. Likewise, not the highest applied concentration was the most advantageous for phenolic components of broccoli sprouts (Gawlik-Dziki et al. 2013).

Putalun et al. (2007) applied three concentrations of yeast suspensions on wormwood hairy root culture, and measured artemisinin content on different days after treatment. The results showed a non-linear relationship between concentrations and artemisinin content. Similar results were seen by Yan et al. (2006) in red sage hairy root cultures.

How many times should spraying be repeated?

Within the reviewed 71 papers, only one deals comparatively with the number of treatments. Zlotek and Swieca (2016) used single and double spraying on lettuce seedlings grown in a growth chamber. Regarding antioxidant power, TPC and ABTS levels benefited from double spraying while DPPH results were higher on single application of yeast extracts. The authors concluded that double sprayed 1% and single sprayed 0.1% extract were the most effective treatments in terms of phytonutrient content, which points the necessity for further comparative studies.

Conclusions

Virtually all of the reviewed 71 studies supported the positive effect of the foliar application of yeast extracts on the nutritional parameters in altogether 38 plant crops, but the role of the actors of this process remained unclear which calls for ongoing research activity. The number of the published comparative studies is limited. As the applied methods show no or minimal commonalities, it is hard to conclude on an ideal combination of concentration, treatment timing, and repetition. At the same time, the great methodological diversity of successful treatments show the power of such interventions, which further stresses the need for basic comparative studies in the following topics, 1., ideal number of treatments to avoid financial losses, 2., ideal timing of treatment(s), and 3., the ideal concentration (and volume) for the highest effect on vegetative and nutritional parameters without any deteriorative impact on cultivated crops and nature. However, the number of *in vitro* studies is increasing; and these, with the involvement of in-depth instrumental investigations, can gradually clarify the questions outlined in this review.

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REVIEW

Probiotic-based cultivation of *Clarias batrachus*: importance and future perspective

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ABSTRACT *Clarias batrachus* (Linn.) is widely recognized in Indian sub-continent for its nutritional and economic significance. At present, it remains at a merely vulnerable state. Pathogenic infections, diminution of natural habitats and introduction of allied exotic fishes are the causes of productivity constraint, particularly in Southern Asia. Conversely, African cat fish *Clarias gariepinus* has been significantly identified as a potential threat to biodiversity, despite being its large scale cultivation across the world. Thus emphasis on indigenous *C. batrachus* farming is becoming inevitable. Currently, screening of autochthonous probiotic organisms for the cultivation of *C. batrachus* in semi-intensive manner is getting importance. At the same time, molecular omics-based technologies are also gaining considerable attention to identify potential probiotic markers. This review provides an overall concept of probiotics, its application and future perspectives in relation to the cultivation of *C. batrachus*.

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Introduction

Aquaculture is becoming a growing and vibrant sector to provide food security to a large population of rural mass. Southern Asia, one of the mega biodiversity hotspots, is native to many indigenous freshwater fish species. *Clarias batrachus* (Linn.) (Asian catfish) is one of the most sought-after aquatic products owing to its nutritional benefits and economic significance though the production of it remains low as per the major carps are concerned. The species is presently on the verge of extinction in Southern Asia due to exploitation of its natural habitats, reclamation of wetlands, uncontrolled introduction of allied exotic fishes and infectious diseases caused by bacterial pathogens (Ahmed et al. 2012). Abrupt use of pesticides in the adjacent agriculture field also has made the situation more hostile. In aquaculture, prevention and control of aquatic diseases by chemical additives or antibiotics may generate antibiotic-resistant bacteria (ARB) and thus creates a serious concern to public health (FAO 2006). The bacterial isolates obtained from a *C. batrachus* population exhibited an increasing order of resistance against antibiotic colistin, ampicillin, gentamycin, carbenicillin, tetracycline, streptomycin, and ciprofloxacin (Pathak and Gopal 2005) that may pose risk to fish fauna and public health (Hoseinifar et al. 2017). Transmission of antibiotic-resistant genes may thus lead to the expansion of pathogenic populations (Bäumler and Sperandio 2016). There are many reports regarding a sharp decrease in productivity due to abrupt use of anti-microbial drugs (Alcaide et al. 2005). Probiotics are beneficial microorganisms which (as ecofriendly and biocompatible substances), are also in increased use to prevent and control aquatic diseases in recent decades. They confer protection against pathogens; e.g., by production of bacteriocins, siderophores, lysozymes and other antimicrobial compound (Ige 2013). Furthermore, they may stimulate immune responses of the host (Bandyopadhyay and Das Mohapatra 2009). Probiotics can be used as a functional feed additive to enhance feed digestibility and fecundity (Hoseinifar et al. 2017).

Scientific approaches along with social and ecological awareness need to be adapted to rescue *C. batrachus* from the existing deplorable state. Semi-intensive mode of cultivation must be prioritized for the conservation of native *C. batrachus* (ICAR-CIFA, 2016-17). Currently, use of autochthonous probiotics is gaining increased importance worldwide to cultivate the species in semi-intensive manner.

Aquaculture probiotics

Probiotics are live microorganisms that confer health benefit to the host when administered in adequate amount (FAO/WHO 2001). A probiotic should be non-pathogenic

and non-toxic to the host and must not contain any virulent or antibiotic-resistant gene (Fuller 1989). It should remain viable without genetic alteration for prolonged periods under storage and field conditions. However, competition for nutrients and production of inhibitory substances could occur even in the rearing water (Dalmin et al. 2001). The acid and bile tolerance property may enable the probiotic organisms to colonize the intestinal tract of the host (De et al. 2014). A probiotic must also exhibit high cell-surface hydrophobicity which ensures its capacity of adherence to the intestinal wall (Krasowska and Sigler 2014). It should be target specific and must reach the desired location (Wang et al. 2008).

Probiotics have been found to play a significant role in the sustainable development of aquaculture through different approaches (Table 1). Aquaculture probiotics do possess different attributes from terrestrial-based probiotics as the gastrointestinal microbiota of aquatic species is affected by the flow of water passing through the digestive tract (Gatesoupe 1999). Commercial probiotics are available both in dry and liquid forms. The dry probiotics (Table 2) consist of spore-forming microorganisms, binding material, a cascade of enzymes coupled with vitamins and other functional additives. The ingredients are then mixed with sterile water for brewing at 27-32 °C for 16 to 18 h with continuous aeration (Sahu et al. 2008). Alternatively, it can directly be added to the feed to use in the same day. However, the hatcheries mostly prefer liquid forms (Table 3) than the dry, spore-forms. Generally, commercial liquid probiotics are extremely hygroscopic and need to be kept away from moisture and sunlight. These liquid forms are applied directly to culture water in the morning and evening and have faster mode of action (Sahu et al. 2008). The immersion method of storing live fish in a probiotic-rich container for certain period of time on a regular basis is also gaining increased attention (Feliatra et al. 2018).

Table 1. Mode of action of aquaculture probiotics.

Mode of action	References
Production of inhibitory substances to the pathogen	Gatesoupe 1999
Competition for adhesion sites and nutrients	Fuller 1989
Improvement in nutrient digestion	Afrilasari et al. 2017
Stimulation of innate immunity	Kim and Austin 2006
Elevates phagocytic activity	Butprom et al. 2013
Growth promotion	Falaye et al. 2016
Influence on water quality	Crab et al. 2010
Stress tolerance	Fuller 1989
Interference in quorum sensing	Defoirdt et al. 2004
Antifungal activity	De et al. 2014
Antiviral activity	Sahu et al. 2008
Protection against infection	Gram et al. 1999
Production of extracellular enzymes	Irianto and Austin 2002
Production of vitamins	Balcazar et al. 2006
Production of siderophores	De et al. 2014
Improvement of host reproduction rate	Ghosh et al. 2004
Improvement of haematological profile	Ayoola et al. 2013
Bioremediation	Devaraja et al. 2013

Molecular tracking of probiotic markers

Molecular identification technologies (e.g., proteomics, transcriptomics, secretomics, metabolomics, interactomics) are getting priority over traditional approaches in recent times to decipher the fundamental basis of probiotics functionality (Papadimitriou et al. 2015). Systematic study of functional genomics is crucial to properly validate putative probionts (Fig. 1). Several attempts have been made to identify molecular markers that would facilitate the rapid screening of probiotic strains. The probiotic must survive at high intestinal bile salt concentration through increased expression of bile stress-regulatory genes (*e.g., bsh*), molecular chaperones (*e.g., GroES, DnaK*), proteases (*e.g., Clps*) or DNA repair proteins (*e.g., uvrB)* (Papadimitriou et al. 2015; Hamon et al. 2014). Tripathy et al. (2014) observed several probiotic marker genes including fibronectin binding protein (*fbp*), mucus binding protein (*mbp*) and bile salt hydrolase (*bsh*) in *Lactobacillus plantarum* KSBT56 strain. The fatty acid biosynthesis (*e.g., fab* gene*)* or quorum sensing (*e.g., luxS* gene) of bacterial strain must be associated with the tolerance to acidic environment (Defoirdt et al. 2004; Hamon et al. 2014). Adhesion is the process of reversible accumulation of bacterial cells belonging to autoaggregation or coaggregation. Probiotics must encode aggregation promoting factor (*e.g.,* Apf), FbpA protein or *adh* gene to colonize and exert antimicrobial (*e.g., albE*) and immune-modulatory (*e.g., slpA*) substances (Papadimitriou et al. 2015). *Bacillus licheniformis*, *Bacillus mycoides*, *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus amyloliquefaciens*, *Bacillus endophyticus*, *Bacillus halodurans*, *Bacillus paralicheniformis* and *Bacillus methylotrophicus* contain class II lanthipeptide that can be identified by the expression of *lanM* gene (Zhao and Kuipers 2016). The genome mining study revealed expression of sublancin 168 and other putative gene clusters of glycocins in *B. thuringiensis*, *B. cereus*, *Bacillus weihenstephanensis*, *Bacillus lehensis*, *Bacillus* sp., *Geobacillus* sp. and *Paenibacillus* sp. (Zhao and Kuipers 2016). *B. thuringiensis, B. cereus* and *Bacillus* sp. BH072 were reported to contain gene clusters of transmembrane protein colicins that depolarize the cytoplasm membrane of pathogen leading to dissipation of

Table 2. Feed probiotics used in aquaculture.

Host	Probiotic	Effect on host	References
Dicentrarchus labrax (European sea bass)	*Debaryomyces hansenii*, *Saccharomyces cerevisiae*	Enhanced growth performance and feed efficiency	Tovar et al. 2002
Epinephelus coioides (Grouper)	*Bacillus pumilus* SE5 and *Bacillus clausii* DE5	Improved growth performance and immune responses	Sun et al. 2010
Ictalurus punctatus (Channel catfish)	*Bacillus* strain	Prevented enteric septicaemia of catfish (ESC)	Ran et al. 2012
Labeo rohita (Rohu)	*Bacillus circulans*	Improved growth performance and feed efficiency	Ghosh et al. 2004
Macrobrachium rosenbergii (Prawn)	*Bacillus subtilis*	Enhanced growth and survivability against pathogenic *Aeromonas hydrophila*	Keysami and Mohammadpour 2013
Oncorhynchus mykiss (Rainbow trout)	*Lactobacillus rhamnosus* JCM 1136	Stimulated immune response	Panigrahi et al. 2005
Oncorhynchus mykiss (Rainbow trout)	*Carnobacterium maltaromaticum* B26, *Carnobacterium divergens* B33	Enhanced the cellular and humoral immune responses	Kim and Austin 2006
Oncorhynchus mykiss (Rainbow trout)	*Bacillus subtilis* AB1	Controlled *Aeromonas* infection	Newaj-Fyzul et al. 2007
Oncorhynchus mykiss (Rainbow trout)	*Aeromonas hydrophila*, *Vibrio fluvialis*, *Carnobacterium* sp.	Enhanced growth performance and feed efficiency	Irianto and Austin 2002
Oncorhynchus mykiss (Rainbow trout)	*Lactobacillus rhamnosus* (ATCC 53103)	Stimulated immune responses	Nikoskelainen et al. 2003
Oreochromis niloticus (Nile tilapia)	*Bacillus subtilis* (ATCC 6633), *Lactobacillus acidophilus*	Stimulated the gut immune system; enhanced the immune and health status; increased the survival rate and body-weight gain	Aly et al. 2008
Oreochromis niloticus (Nile tilapia)	*Streptococcus faecium*, *Lactobacillus acidophilus*, *Saccharomyces cerevisiae*	Increased growth, digestibility and feed conversion ratio	Lara-Flores et al. 2003
Penaeus monodon (Asian tiger shrimp)	*Bacillus* sp. S11	Enhanced growth performance and feed efficiency	Rengpipat et al. 1998
Salmo salar (Atlantic salmon)	*Carnobacterium divergens* 6251	Inhibited *Aeromonas salmonicida* and *Vibrio anguillarum*-induced pathogenicity	Ringo et al. 2007
Salmo salar (Atlantic salmon)	*Carnobacterium* sp.	Inhibited *Aeromonas salmonicida*, *Vibrio ordalii*, *Yersinia ruckeri* and reduced disease outbreak	Robertson et al. 2000

cellular energy (Zhao and Kuipers 2016). Probiotic often contain genes (*e.g., tasA, tapA, bslA*) to synthesize biofilms by secreting extracellular matrix protein. *Bacillus subtilis* biofilms is synthesized by the products of the *eps* gene (Papadimitriou et al. 2015). Several gene or protein markers were involved in biofilm formation (*e.g., bslA* and *tapA*), quorum-sensing (*e.g., PlcR*) in *B. cereus* and *B. thuringiensis* (Majed et al. 2016). *sipW*, *tasA*, and *calY* transcriptions were repressed by the SinR regulator which controls biofilm formation through production of kurstakin; a lipopeptide biosurfactant (Majed et al. 2016). The further identification of molecular markers including housekeeping genes can enrich our understanding about the probable mode of action of probiotics.

The Asian catfish *Clarias batrachus*

Asian catfish *C. batrachus* has a broad depressed head covered with bony plates, the snout of which contains four pairs of sensory barbels (Jayaram 1981). The skin mucus often contains bactericidal proteins and provides protection against invading pathogens (Elavarasi et al. 2013). The body, generally grayish-black, is cylindrical and tapers towards the caudal peduncle. *C. batrachus* typically attains a standard length of 225-300 mm. However, in India it is found to be around 183.1 mm in length as an average (Ng and Kottelat 2008).

The nutritional profile of *C. batrachus* contains easily digestible high-grade protein (16.26 g/100 g), iron (2.20 mg/100 g), minerals, good cholesterol and polyunsaturated fatty acids (ICAR-CIFA). It is also a rich source of vitamin A (6.03 IU/100 g), vitamin D (44.73 IU/100 g) and essential amino acids (ICAR-CIFA; Mohanty et al. 2014).

C. batrachus can be found in both fresh and brackish water of Sri Lanka, India, Pakistan, Bangladesh, China, Burma, Malaya, Singapore, Philippines, Borneo, Java, and Thailand (Talwar and Jhingran 1991). They can be found in a variety of habitats, most commonly in muddy or swampy low-land field and rice fields. The major constraint in the cultivation of *C. batrachus* in natural resources is the non-availability of quality seeds. The scarcity of seeds arises from various factors like: indiscriminate use of pesticides in paddy fields, industrial effluents, diminution

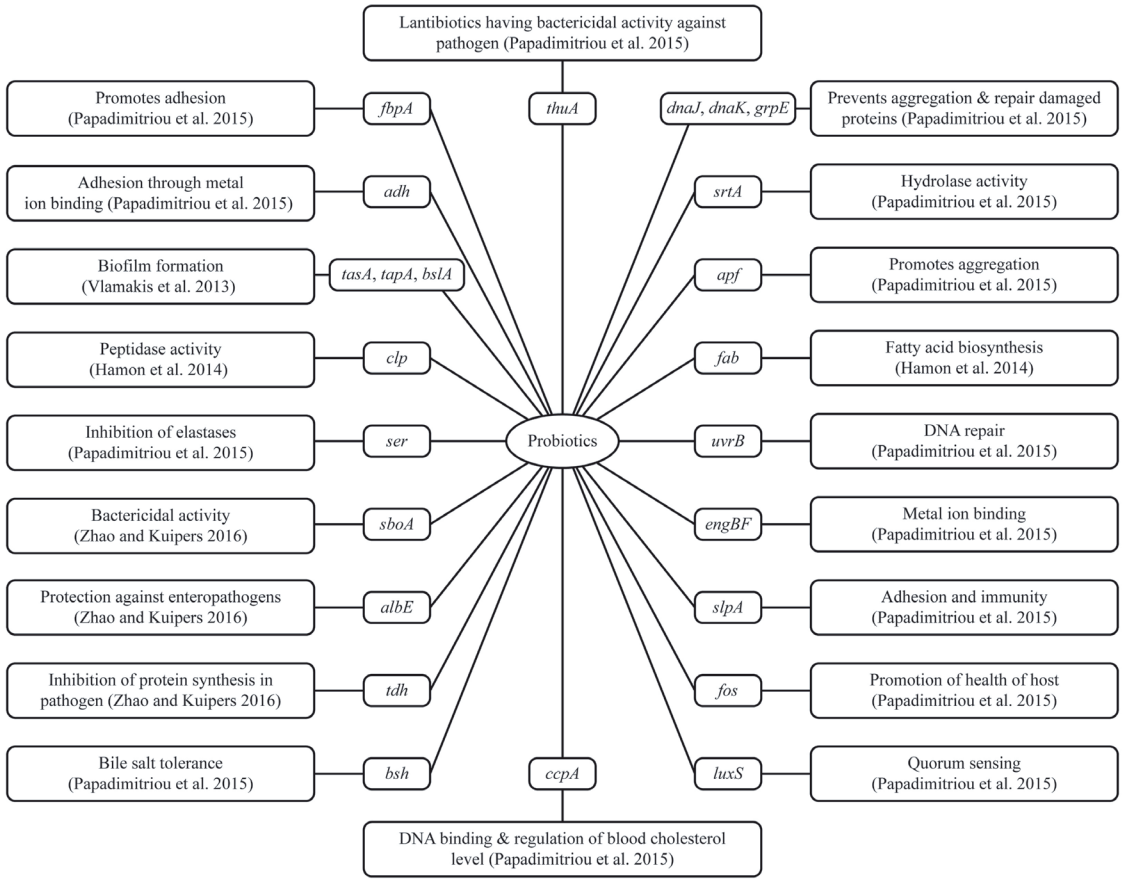

Figure 1. Genes related to potential probiotic properties.

of breeding area due to siltation, intermittent periods of drought and illicit fishing of juveniles and brood fishes (Dhara and Saha 2013). The exotic catfish *C. gariepinus* that has morphological resemblance to indigenous *C. batrachus* is frequently misled by some dishonest traders (Khedkar et al. 2015). These have threatened the mere existence of this indigenous catfish. Therefore, it becomes crucial to carry out breeding and rearing of *C. batrachus* to meet the need of the society. As the species is sold only in living condition and cannot be transported over long distances, a culturing in semi-intensive manner becomes necessary. Thus, cultivation in small production pond and its supply to the market in living condition would be a better practice for catfish farming.

Probiotics in *Clarias* species

The invasive alien catfish *C. gariepinus* has presently been considered as a potential threat due to its frenzied feeding behavior and hence, farming of indigenous *C. batrachus* is regaining its importance (Radhakrishnan et al. 2011). However, reports on the use of probiotics in *C. gariepinus* are available, but our knowledge is still limited so far as *C. batrachus* (Table 4) are concerned. In this regard, a protocol to screen putative probiotic strain for cultivation of *C. batrachus* can be proposed (Fig. 2).

Probiotic strains usually synthesize extracellular enzymes (e.g., proteases, amylases, lipases) and growth factors (e.g., vitamins, fatty acids, amino acids) which can stimulate the appetite and endorse fish nutrition by the detoxification of toxic substances and breakdown of indigestible components (Irianto and Austin 2002; Balcazar et al. 2006). Consequently, nutrients are more readily absorbed when the feed is supplemented with probiotics (Afrilasari et al. 2017). The use of *Lactobacillus acidophilus* with a diet for 12 weeks has exhibited improved specific growth rate (SGR), relative growth rate (RGR), protein efficiency ratio (PER), feed conversion ratio (FCR), haematological parameter and significantly ($p < 0.05$) higher survival rate (SR) in *C. gariepinus* fingerlings (Ige 2013). Banerjee and co-workers (2015) isolated an extracellular enzyme-producing bacterial strain *Bacillus licheniformis* from *C. batrachus*. Dey et al. (2016) obtained an extracellular enzyme-producing autochthonous gut bacteria *B. cereus* HG01 (KR809412) from *C. batrachus*. Ayo Olalusi et

Table 3. Examples of water probiotics used in aquaculture.

Host	Probiotic	Effect on host	References
Litopenaeus vannamei (Pacific white shrimp)	*Bacillus* sp. and *Lactobacillus*	Improved the environmental quality of the sediment and water in ponds with closed recirculation systems	Paiva-Maia et al. 2013
Oncorhynchus mykiss (Rainbow trout)	*Pseudomonas fluorescens* AH2	Increased survival rate against pathogenic *Vibrio anguillarum*	Gram et al. 1999
Penaeus monodon (Asian tiger shrimp)	*Bacillus* sp.	Improved growth and survival rate, maintained water quality	Dalmin et al. 2001
Penaeus monodon (Asian tiger shrimp)	*Bacillus pumilus*, *B. licheniformis* and *B. subtilis*	Reduced total ammonia nitrogen (TAN); improved growth and survival rate	Devaraja et al. 2013
Penaeus vannamei (White shrimp)	*Bacillus* sp., *S. cerevisiae*, *Nitrosomonas* sp.	Reduced concentrations of nitrogen and phosphorus, increased yields of shrimp	Wang et al. 2005
Scophthalmus maximus (Turbot larva)	Lactic acid bacteria	Increased survival rate against vibriosis	Gatesoupe 1994
Scophthalmus maximus (Turbot larvae)	*Roseobacter* sp.	Improved survival rate	Hjelm et al. 2004

al. (2014) showed that viable feed-probiotics administered to *C. gariepinus* increased the hematological parameter and digestive enzyme (amylase and lipase) activity of the catfish within acceptable range. Probiotic *L. plantarum* infused diet considerably enhanced hematological parameters, carcass protein and mineral composition of African catfish *C. gariepinus* (Nwanna and Tope-Jegede 2016).

A higher level of immunity was also noted while challenging with pathogenic *Salmonella typhi* than those with non-probiotic diet. Probiotic *Bacillus aryabhattai* KP784311, *B. flexus* KR809411, *B. cereus* KR809412 encapsulated chironomid midge larvae significantly ($p < 0.05$) increased specific growth rate and survivability of *C. batrachus* (Dey et al. 2017).

Synergistic relationships among different bacterial strains may be more effective and consistent than a single strain of probiotic (Salinas et al. 2008). *L. plantarum* and *Pseudomonas fluorescens* have a synergistic effect on each other and therefore produced higher specific growth rate and survival rate in *C. gariepinus* fingerlings than the control diet (Omenwa et al. 2015). Ayoola et al. (2013) reported that administering a mixture of *Lactobacillus* and *Bifidobacterium* species in a feeding trial (90 days) enhanced feed efficiency, growth rate, survivability and nutritional quality of *C. gariepinus* juveniles. The hydrobiological parameters are also important in maintaining the integrity of aquatic ecosystem and have direct influence on the productivity of *C. batrachus* (Ganguly et al. 2017).

Biofloc technology is a sustainable method to meliorate water quality and feed utilization efficiency of aquatic animals (Crab et al. 2010). The fermented biofloc inoculated with the bacterium *B. cereus* enhanced the growth and feed utilization efficiency of juvenile catfish *C. gariepinus* (Hapsari 2016). Putra et al. (2017) also reported to achieve better growth performance and feed utilization efficiency in African catfish *C. gariepinus* using biofloc technology infused with the *Bacillus* probiotic.

Probiotic microorganisms often exert bactericidal or bacteriostatic substances to restrict the propagation of pathogenic bacteria (Sahu et al. 2008). Kato et al. (2016) have conducted a study to isolate and identify probiotic bacteria from the surface of the African catfish *C. gariepinus.* Among all the isolates, *Lactococcus sp.* and *Lactobacillus sp.* have shown potential antimicrobial activity against selected pathogenic strains. *Lactobacillus fermentum* LbFF4 and *L. plantarum* LbOGI isolated from *C. gariepinus* showed *in vitro* antibacterial activities against gram negative bacteria *Citrobacter, E. coli, Klebsiella*, *Proteus*, *Pseudomonas* and *Salmonella* (Ogunshe and Olabode 2009). Fortified diet enriched with *L. plantarum* enhanced growth, weight gain and FCR of cultured *C. gariepinus* fingerlings (Falaye et al. 2016). A parallel study with *C. gariepinus* showed increased growth performance, FCR, PER, protein productive value and energy retention when the fish feed was supplemented with a commercial probiotic strain of *Bacillus* (El-Haroun 2007). The organism germinated in the intestine and synthesized digestive enzymes amylase, protease and lipase which in turn contributed improved feed efficiency.

Probiotics often exert immunomodulating substances to stimulate immune response against pathogenic invasion (De et al. 2014). *Vibrio anguillarum, Vibrio alginolyticus* and *Aeromonas hydrophila* were reported to cause pathogenicity in *C. batrachus* (Ahmed et al. 2012). Dahiya et al. (2012) successfully experimented with *C. batrachus* fingerlings, treating them with probiotics that resulted remarkable increase of immunity and hemoglobin level of the catfish. *L. plantarum* C014 infused (10^7 cfu/g) diet improved innate immune response and disease resistance ability of hybrid catfish. The probiotic-supplemented diet elevated

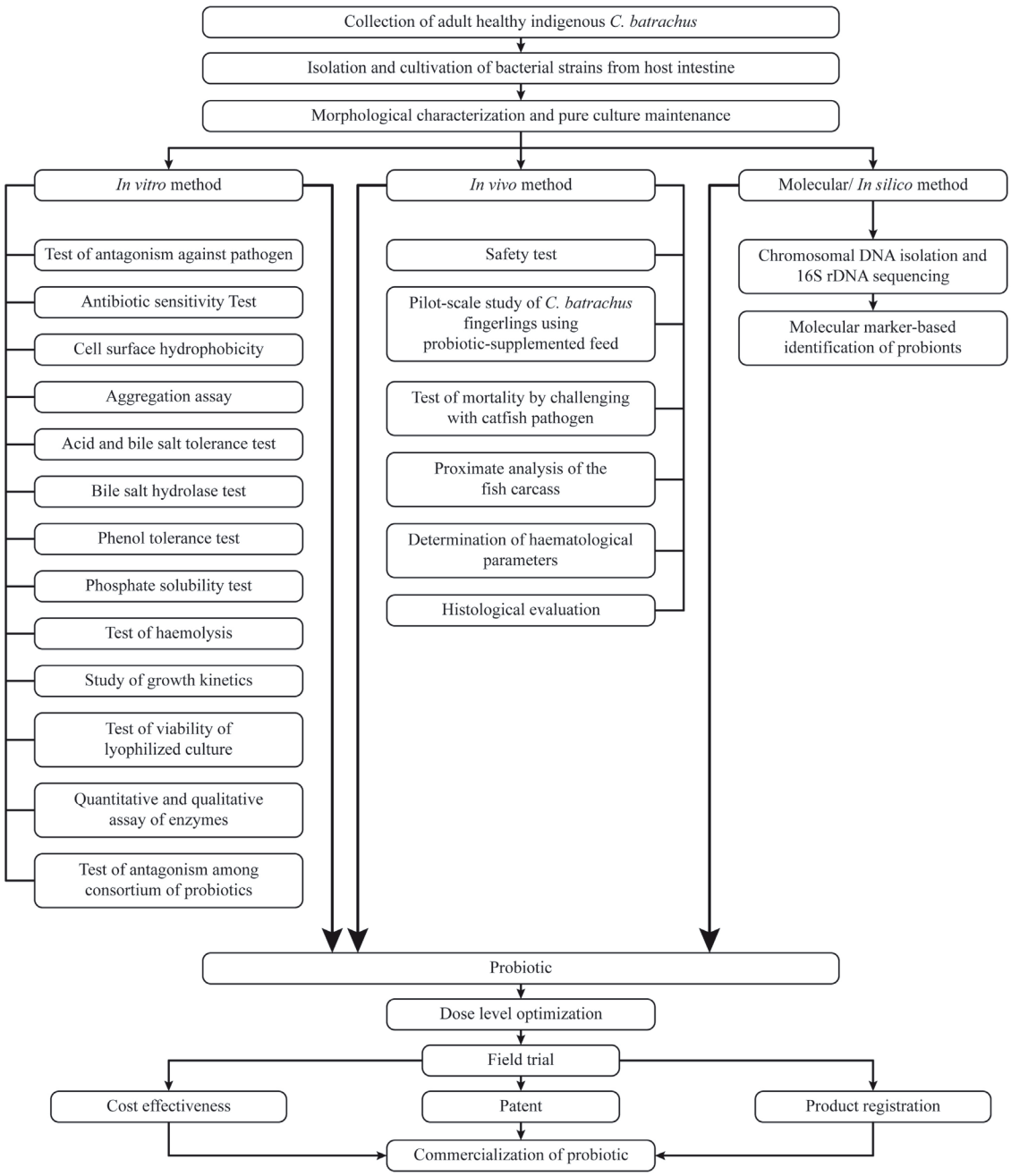

Figure 2. Diagram for screening of autochthonous probiotic strain for the cultivation of *C. batrachus*.

phagocytic activity, lysozyme efficacy and survival rate of hybrid catfish against *A. hydrophila* infection (Butprom et al. 2013).

Probiotic microorganisms resist the establishment of pathogen by adsorbing and colonizing the digestive tract of the host (Fuller 1989) through a process called competitive exclusion. One experiment was carried out to evaluate the effects of the probiotic bacterium *Bacillus megaterium* PTB 1.4 on the growth performance, intestinal microflora and digestive enzyme activity of *Clarias sp.* (Afrilasari et al. 2017). The results showed significantly higher ($p < 0.05$) SGR and increased activity of protease and amylase in fish maintained on the probiotic-supplemented diet compared to those on the control diet. Bairagi et al. (2002) isolated distinct microbial source of digestive enzymes amylase, lipase and protease from the gastrointestinal tract of *C. batrachus* that may contribute towards better feed formulations. Yakubu et al. (2016) assessed the effects

of commercial probiotic (Biogut) on *C. gariepinus* and observed improved growth and survival rate of the fry. Jahan et al. (2016) noticed better growth performance, SGR and proximate carcass compositions of *C. batrachus* fingerlings fed with probiotic-supplement diet compared to the control diet.

Quorum sensing is a bacterial cell-to-cell communication mechanism leading to the alternation of gene expression in response to high population density (De Almeida et al. 2016). The quorum quenching or disruption of quorum sensing is considered as potential anti-infective strategy in aquaculture (Defoirdt et al. 2004). The probiotic *Lysinibacillus sphaericus*, *B. amyloliquefaciens* and *B. cereus* have been reported to disintegrate acyl homoserine lactone (AHL), the quorum sensing molecule of pathogenic *A. hydrophila* by producing AHL-lactonase and thus preventing motile aeromonad septicemia (MAS) in *C. gariepinus* (Novita et al. 2015).

Conclusion

In the era of global food crisis, aquaculture stands as a sustainable approach to restore biodiversity and ensure nutritional security (Naylor et al. 2000). Countries where a large section is battling with protein deficiency and malnutrition, consumption of *C. batrachus* may hold promising potentiality in uplifting the overall health status of the populace. To reinstate the genetic resources of *C. batrachus*, semi-intensive aquaculture practices has to be adopted. A concerted effort is the need of the hour to cultivate *C. batrachus* due to its apparent nutritional and economic significance, whereas rational selection and proper validation of probiotic is a cause of concern. The limnological properties of aquatic pond may pose a threat to the establishment of probiotics and thus affect fish health. The laboratory result with test probiotics should be in accordance with large-scale commercial implication. The preservation of probiotics maintaining the viability of the organisms is yet to be standardized. The dose-level optimization of a certain probiotic strain also needs to be carried out before commercialization.

However, different molecular technique-based approaches (polymerase chain reaction, multiplex-PCR, pulsed field gel electrophoresis, random amplified polymorphic DNA, fourier-transform infrared spectroscopy, denaturing gradient gel electrophoresis, temporal temperature gradient gel electrophoresis, fluorescence *in situ* hybridization) are now being increasingly used for the analysis of GI microflora to screen autochthonous

Table 4. Application of probiotics in *Clarias* species.

Host	Probiotic	Effect on host	References
Clarias batrachus (Asian catfish)	*Lysinibacillus sphaericus*	Inhibited *Vibrio harveyi* infection	Ganguly et al. 2018
Clarias batrachus (Asian catfish)	*Lactobacillus sporogenes*, *Saccharomyces boulardii*	Controlled *A. hydrophila* infection	Dahiya et al. 2012
Clarias batrachus (Asian catfish)	*Nitromonas, Rhodococcus*, *Bacillus megaterium*, *Lecheni formis*, *Desulphovibrio sulphuricum*, *Pseudomonas*, *Chromatium*, *Chlorobium*, *Thiobacillus thioxidants*, *Thiobacillus ferroxidant*, *Methylomonas metyhanica*, *Glucon acetobactor*, *Azospirillum*, *Trichoderma*, *Shizophyllum commune*, *Sclertium gluconicum*	Increased haematological profile, inhibited *A. hydrophila* infection	Dahiya et al. 2012
Clarias gariepinus (African catfish)	*Lactobacillus acidophilus*	Improved growth, survivability, feed efficiency	Al-Dohail et al. 2009
Clarias gariepinus (African catfish)	*Lactobacillus acidophilus*	Enhanced haematology parameters, stimulated immunity, inhibited *Staphylococcus xylosus*, *Aeromonas hydrophila* gr.2 and *Streptococcus agalactiae* infection	Al-Dohail et al. 2011
Clarias gariepinus (African catfish)	*Bacillus thuringiensis*	Enhanced of cellular non-specific immune response against *A. hydrophila* infection	Reneshwary et al. 2011
Clarias gariepinus (African catfish)	*Lactobacillus acidophilus*	Improved fish health, enhanced haematological parameters	Olayinka and Afolabi 2013
Clarias gariepinus hybrid (MCF♀ × QCF♂) (Egyptian African catfish)	*Saccharomyces cerevisiae*	Increased body weight and growth rate	Essa et al. 2011
Clarias gariepinus (African catfish)	*Lactobacillus acidophilus*, *Bacillus subtilis*, *Lactobacillus bulgaricus*	Enhanced growth and survival rate of African catfish larva	Dennis and Uchenna 2016
Clarias orientalis (Catfish)	*Lactobacillus* sp.	Increased growth and survival rate, inhibited *Aeromonas* and *Vibrio* sp.	Dhanasekaran et al. 2008

probiotics (Kim et al. 2007). The 'omics' studies may also provide a potential opportunity to obtain probiotic microorganisms avoiding traditional cultivation methods. The use of probiotics to potentiate the benefits of *C. batrachus* stand necessary and its application is both empirical and scientific. A futuristic approach with probiotics maintaining ecologically sound management practices has to be adopted to bring about socio-economic upliftment in Asian countries which are the native place of *C. batrachus*.

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ARTICLE

Bioelectricity production in an indoor plant-microbial biotechnological system with *Alisma plantago-aquatica*

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ABSTRACT The paper descibes the development of a biotechnological system for generating bioelectricity on closed balconies of buildings from living plants *Alisma plantago-aquatica* and soil microorganisms grown in containers with natural wetland substrate, provided with a graphite and Zn-galvanized steel electrode system. This biotechnology worked efficiently from the first days after installation and was practically at full capacity 2 weeks later. Electric power output was highest in the spring-summer and the early autumn period (at the time of the highest photosynthetic activity of plants). The highest current output was 58.6 mA at 10 Ω load. Bioelectricity generation by the biosystem was stable with slight fluctuations throughout the year in well-lighted and heated premises at a temperature of 21-26 °C, and the seasonal reduction of the bioelectricity level was 8.71%. On not-heated closed terraces and glazed balconies, with temperature fluctuations from 5 to 26 °C, the electricity production decreased in the winter period by 19.98% and 39.91% with and without adding of sulfate-reducing bacteria, respectively. The proposed system of electrodes for collection of bioelectric power is new, easy to manufacture and economical. It is resistant to waterlogged environment, and has good prospects for further improvements for more effective collection of plant-microbial bioelectricity. Maintainance of the biosystem is simple and accessible to everyone without special skills.
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Introduction

Getting electricity from living plants and microorganisms directly from their place of living, i.e. from the soil, is an innovative method of alternative energy production, which has been actively investigated by scientists in the last 10 years (Strik et al. 2008; De Schamphelair et al. 2008; Kaku et al. 2008; Helder et al. 2010; Picot et al. 2011; Rothballer et al. 2011; Kuijken et al. 2011; Timmers et al. 2012; Liu et al. 2013; Lu et al. 2015, Koen et al. 2015; Rahimnejad et al. 2015; Moqsud et al. 2017; Nitisoravut et al. 2017).

The project "Starry Sky" of Plant-e Company (Wageningen, Netherlands) is the first real example of implementation of the plant-microbial power generation with 300 roadside LED bulbs lighted by electricity produced by the plant-microbial groups of the surrounding soil near Amsterdam in the Netherlands (Schultz 2014).

The energy of the electrons emitted by soil bacteria during the decomposition of the root excretions of plants is the source of electricity in this biotechnology (Strik et al. 2008; Nitisoravut and Regmi 2017). Thus, the green cover on the roof of a building (or around) solves the problem of its energy supply and at the same time provides ecological coverage of the building or landscaping of its surroundings. With the improving technology, 100 m^2 of vegetation of a green roof will be able to provide energy for a house that consumes 2800 kWh per year (Strik et al. 2011). The technology of producing electricity from living plants and microorganisms is prospective for a widespread application of energy supply to buildings, street lighting, WiFi access points, mobile phone charging, and various energy demands of ecotourism (Helder et al. 2012; Wetser et al. 2015).

Bioelectricity from root excretions of living plants and soil microorganisms is renewable and environment friendly. Its exploitation does not cause emission of greenhouse gases or toxic substances because the technology is aimed only at collection of the electrons circulating in the substrate through introducing a system of electron collection into it (Strik et al. 2008). In addition, green roofs contribute to preservation of the environment since they significantly reduce energy consumption by the building (Castleton et al. 2010).

Nowadays, the main problems of plant-microbial bioelectric technology are the low power and high cost of materials used to collect the electrons and protons (Helder et al. 2013a; Behera and Varma 2016). However, there are

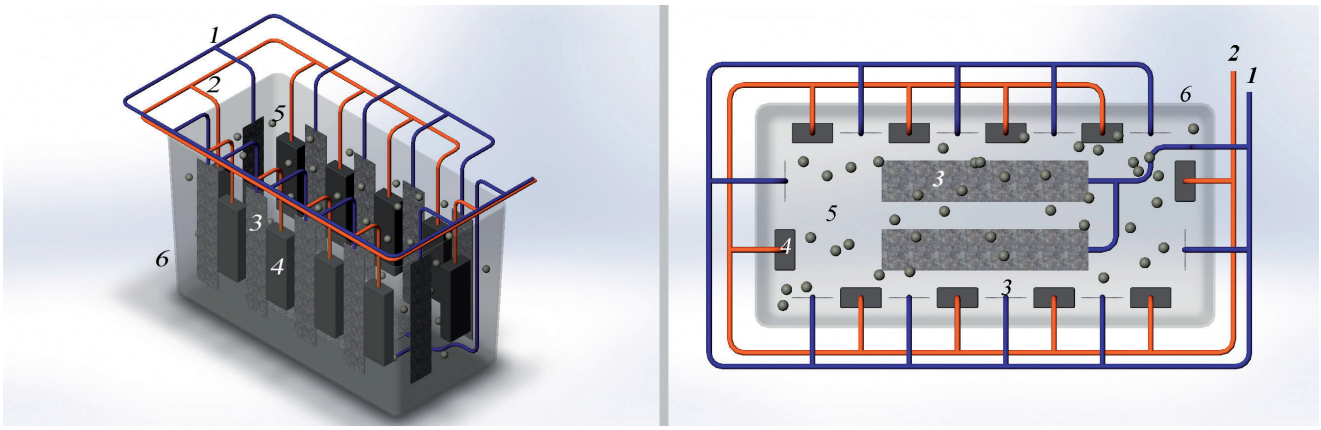

Figure 1. Schematic arrangement of the system of electrodes in container with the plants. A: front side view of the container. B: top view of the container. 1 - output wire, connecting the system of 12 anodes; 2 - output wire, connecting the system of 10 cathodes; 3 - anode; 4 - cathode; 5 - substrate; 6 - container; 7 - plants.

many ways to increase the capacity of plant-microbial electrosystems, e.g., improvement of the cathodes and anodes (Picot et al. 2011; Chen et al. 2012; Helder et al. 2012; Nitisoravut et al. 2017; Wetser et al. 2017), experiments with new environments (Timmers et al. 2010; Helder et al. 2011; Lu et al. 2015; Moqsud et al. 2015) and with new plants (De Schamphelaire et al. 2008; Kaku et al. 2008; Helder et al. 2010; Timmers et al. 2010; Timmers et al. 2012; Hubenova and Mitov 2012; Yadav et al. 2012; Liu et al. 2013; Lu et al. 2015; Oon et al. 2015; Wetser et al. 2015; Regmi 2016) have been published. In this study, our aim was the improvement of this technology by developing and testing a new biosystem, with new cheap electrodes, a non-standard environment, and new species of plant.

Seasonal reduction of power energy produced on green roofs is another problem of using the energy of living plants and microorganisms (Helder et al. 2013b). The decline of power output in the winter-spring period under outdoor conditions has been well documented (Helder et al. 2013b; Daj et al. 2015); power received from green roofs and marsh areas was significantly lower than that obtained in the laboratory. We decided to test the possibility of using indoor premises: closed and glazed terraces, balconies or winter gardens, as an alternative to green roofs in countries with extremely cold or arid climate to prevent seasonal losses of power energy for year-round production of plant-microbial energy.

To implement the above-written aims, we set up the following tasks:

- select plants that could be grown inside buildings (in glazed terraces or balconies or near the windows of the apartments) while producing electricity;
- explore the possibility of their long-term cultivation in enclosed conditions of buildings;
- assess the prolonged functionality of the electrode system in aquatic environment;
- determine the current and voltage output that can be obtained from our bioelectric system and assess its suitability for the production of bioelectricity inside buildings for a long time;
- estimate the effect of external factors on the generation of bioelectricity on green indoor balconies and terraces.

Materials and Methods

Biocomponents of the electro-biotechnological system

Spring is the optimal time for replanting of *Alisma* (Grigoriev et al. 2006), the plant chosen by us as a biocomponent for the electro-biotechnological system. Plant specimens were taken from various city reservoirs, ponds and lakes, in the city of Lviv (Ukraine). Young specimens with 2-3 small leaves that have not yet blossomed were taken and planted into swampy soil substrate in plastic containers of 0.0525 m^2 area with a system of electrodes to collect electricity. The substrate for plant cultivation consisted of silt from a natural reservoir and water in a proportion of 2:1. Each box contained two bushes of plants. The plants were not disinfected, not to break the original relationship of microorganisms and plant roots, crucial for generating electricity.

The 100 ml suspension of *Desulfovibrio* sp. Yav-6 (Moroz and Rusyn 2012) with cell density of 2.7 mg/ml was added to some containers two times during the plant development (in the 1st and 2nd month) to test the hypothesis of favorable influence of sulfate-reducing bacteria on the level of bioelectricity.

Plant growth was measured by counting and summing the lengths of leaves and stems to obtain total length

(Helder et al. 2010); average values were calculated per container.

Electrical components of the system

Our bioelectricity collecting and measuring system (Rusyn and Medvediev 2018) consisted of electrodes placed in the plastic container with plants, directly in the zone of association of plant roots and microorganisms, where electrons and protons are released. For anodes, galvanized steel plates of 292(h) x 30(w) x 0,8(t) mm size were used. For cathodes, graphite plates of 90(h) x 30(w) x 15(t) mm size (Rusyn and Medvediev 2015) were prepared. Multi-conductor copper wires of 1.5 mm diameter were used for connection of cathodes and anodes (Rusyn and Medvediev 2016).

The anode system of one container consisted of 12 plates, connected by wires and located in the container at a distance of 0.5 cm from its walls as follows: 2 plates were put to the bottom of the container and 10 plates, which bent on third part to the form of a one entire plane, were placed vertically along the walls (Fig. 1A, B).

The system of cathodes consisted of 11 graphite plates connected by wires and located in the container vertically between the anodes (Fig. 1A, B) at a depth of 10 cm and at a distance of 0.5 cm from the vertical walls of the container.

Experiment design

The containers with plants and electrodes were kept inside the buildings in different conditions:

1. on unheated glazed terraces and balconies, where temperatures ranged from 5 °C in winter to 26 °C in summer;

2. on glazed balconies and terraces heated in winter, at average temperature from 21 °C to 26 °C for the duration of the experiment, and under identical conditions, directly in apartments with temperature of 21 °C to 26 °C throughout the time.

The experiment was conducted in realistic conditions that can be created in private appartments, and in two different temperature regimes as the the housing stock consists of two types of enclosed balconies and terraces: heated and without heating. Variable mixed lighting, including natural illumination by daylight through the window and artificial light sources, was applied. The plants were watered every 2-3 days.

The experiment with repetitions continued for 40 weeks for a few years from the spring planting to the early spring of next year, when it was possible again to renew the vegetation by planting new plants, to reveal the peculiarities of the development of plants and of the generation of bioelectricity in enclosed living quarters.

Measurements and calculations

The values of voltage (bioelectric potential; U, mV) and current (I, mA) were measured with a digital multimeter, with the probes connected to the wires coming from the cathodes and anodes. Measurements were performed daily.

Open circuit voltage (open bioelectric potential) was monitored over time in open-circuit state of the plant-microbial biosystem.

In order to determine at which external resistance one can obtain maximum power density, measurements were carried out using different loads from 10 Ω to 12 kΩ. Resistors of 10, 50, 250, 500, 1000, 3000, 5000, 12000 Ω were used, and the voltage through the external resistor, connected in the circuit periodically for 15 min, was recorded. During a laboratory experiment, the voltage was also measured when an external resistor was permanently connected for several days. Current strength was calculated theoretically using practically measured voltage and resistance, but was also in fact measured, with resistors.

Current was calculated as the voltage output divided by external resistance according to equation

$$I\,(A) = U\,(V) / R\,(\Omega)\ \text{(Ohm's law)}$$

and the current density was calculated as

$$J\,(A/m^2) = I\,(A) / S\,(m^2),$$

where U is the measured voltage; R, the external resistance; and S, the active area of the electrodes. Power density was calculated as

$$P\,(W/m^2) = J\,(A/m^2) * U\,(V)$$

Power density was normalized to the $1 m^2$ experimental planting surface of the plants covered by the electrodes. The average bioelectric potential and current strength were calculated two-weekly and for the total 40 weeks of the experiment.

Results and Discussion

Selection of plants for electric biotechnology in enclosed indoors areas

The use of *Oryza sativa* (Kaku et al. 2008; De Schamphelaire et al. 2008), *Arundinella anomala*, *Arundo donax* (Helder et al. 2010), *Spartina anglica* (Timmers et al. 2010; Helder et al. 2010; Wetser et al. 2015), *Glyceria maxima* (Timmers et al. 2012), *Ipomea aquatica* (Liu et al. 2013) and *Phragmites australis* (Wetser et al. 2015) in the technology of bioelectricity production from living plants has been described in literature. The choice of plant for electric biotechnology is very important and determines how effective it will work. When applying the same systems of collecting electricity, but using different plants, energy output differed tenfold: the power obtained from *Spartina anglica* and *Arundinella anomala* was 0.222 W/m^2 and 0.022

Table 1. The development of *A. plantago*-aquatica plants in the biosystems on unheated and heated terraces and balconies without or with the addition of sulfate-reducing bacteria *Desulfovibrio* sp. ($p < 0.05$).

Time of plant growth (week)	Conditions					
	5 - 26 °C / unheated premises / without sulfate-reducing bacteria	5 - 26 °C / unheated premises / with sulfate-reducing bacteria	21 - 26 °C / heated premises / without sulfate-reducing bacteria	5 - 26 °C / unheated premises / without sulfate-reducing bacteria	5 - 26 °C / unheated premises /with sulfate-reducing bacteria	21 - 26 °C / heated premises / without sulfate-reducing bacteria
	The average number of leaves in one biosystem			Quality of plants		
0	6.1	5.3	6.0	***	***	***
2	11.7	10.2	11.2	***	***	***
4	14.9	15.6	16.3	***	***	***
6	16.7	16.8	17.6	***	***	***
8	16.6	17.5	17.8	***	***	***
10	15.9	17.4	18.4	***	***	***
12	16.7	19.8	19.5	***	***	***
14	14.5	18.5	18.3	**+	***	***
16	9.3	17.1	17.4	**+	***	***
18	6.2	16.7	17.2	*++	**+	***
20	4.1	10.6	17.7	*++	*++	**+
22	2.3	5.5	16.6	+++	+++	***
24	1.1	1.3	16.8	+++	+++	***
26	0	0	17.8	-	-	***
28	0	0	17.3	-	-	***
30	0	0	17.6	-	-	***
32	0	0	16.7	-	-	***
34	0	0	17.1	-	-	***
36	0	0	17.0	-	-	***
38	0	0	16.5	-	-	***
40	0	0	16.9	-	-	***

*** Green leaves. +++ Yellow and drying leaves. - Death of all plants

W/m^2, respectively (Helder et al. 2010).

The plants mentioned above are plants of wet habitats. We also focused our attention on the wetland plants when choosing plants for bioelectric power generation and, simultaneously, landscaping of buildings. It is precisely in waterlogged conditions of wetlands where bioelectricity, produced by plants and microorganisms, can be most fully exploited, because penetration of oxygen to the electrodes and the consequent loss of circulating electrons and protons is reduced to a minimum (Helder et al. 2012), and as wetlands are also ideal for the development of electricity producing microorganisms (Lovley et al. 2011).

The plants for bioelectric technology were selected among not demanding plants that do not require special care, are cosmopolitan and decorative, and at the same time perennial. Perennials, compared to annual plants, have a more active secretion of organic substances in the soil (Lynch and Whipps 1990; Kuzyakov and Domanski 2000), which leads to the accumulation of the so-called rhizodeposit in the root zone of the soil (Dennis et al. 2010), and production of bioelectricity depends on splitting of rhizodeposit by the microorganisms.

Alisma plantago-aquatica, also known as European water-plantain or common water-plantain (Crocker and Wilmer 1914; Tsvelev 1979) was chosen as a plant that possesses all above-mentioned necessary characteristics for bioelectricity generation technology. It is a perennial grassy plant, up to 0.6 - 1 m high (Gubanov et al. 2002; Grigoriev et al. 2006), which suggests the presence of ample rhizodeposit. The plant is used in landscape design; it is planted along ponds or in marshy areas of gardens and parks (Grigoriev et al. 2006). In spring and early summer, the plant is attractive by its rosettes of leaves, and in summer, with its small white, sometimes pink or lilac flowers that appear on long stems (Darbyshire et al. 2014). Water plantain practically needs no care. It is widespread in all continents, both in the northern and southern regions, including Africa and Australia (Grigoriev et al. 2006). Thus, *A. plantago-aquatica* was selected as a plant for bioelectricity generation.

Cultivation of A. plantago-aquatica in bioelectric power generation systems in buildings on closed balconies and terraces

Our next task was to find out if it is possible to cultivate *A. plantago-aquatica* indoors in containers with systems of electrodes. The plant was found to be able to grow in typical conditions on unheated enclosed balconies and

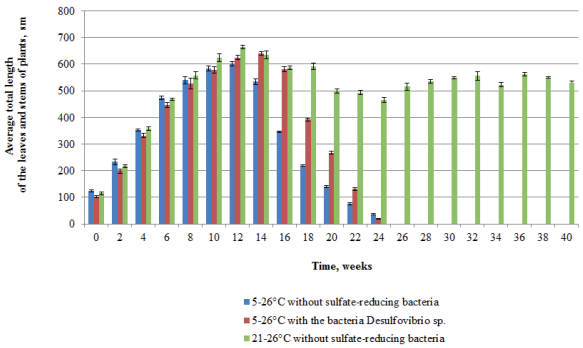

Figure 2. Growth of *Alisma plantago-aquatica* in bioelectricity systems indoors under different conditions during 40 weeks of the experiment: the average total length of leaves and stems of the biosystem plants during sudden temperature fluctuations from 5 to 26 °C with and without addition of bacteria *Desulfovibrio* sp., and at stable temperature range of 21 - 26 °C.

terraces in a marshy substrate in containers with systems of anodes and cathodes from early spring to mid-autumn, and all year round on the heated terraces and balconies or directly in the flats (Fig. 2).

The results of monitoring the plant growth during the spring-summer period under conditions of closed premises showed that such an environment was favorable for development of plants: new leaves grew actively, the number of leaves in the containers nearly doubled during the first two weeks, in the next two weeks the quantity of leaves yet increased by 41.9% on the average (Table 1). Leaf biomass increased during summer, and the plants bloomed (Fig. 3A-D).

In the autumn period, temperature decrease and reduction in daylight on unheated terraces caused the leaves to turn yellow and wilt and, despite the appearance of young shoots, the plants died (Fig. 3E, F and Table 1). In systems with added sulfur-reducing bacteria, the plants retailed the leaves for a longer period, and the yellowing and wilt of leaves took place 4 weeks later than in the containers without bacteria (Table 1). At that time, the plants in containers without added bacteria lost about half of foliage mass, and the leaves turned yellow and dried up.

Displacement of containers with plants to warm and well-lit areas of the flats from early autumn to early spring prevented the decay of plants and they continued to develop the whole winter (Table 1). Year-round cultivation of water plantain is possible on enclosed heated balconies and terraces, and near windows in the apartments, where the temperature does not fall (Fig. 2). *A. plantago-aquatica* reproduces by seeds and division of rhizomes (Gubanov et al. 2002). Strong self-seeding throughout the summer and germination of young bushes from rhizomes provide year-round green cover of containers. System maintenance

Figure 3. The development of plants *A. plantago-aquatica*, transferred from the natural reservoirs in May in the containers with muddy substrate and a system of electrodes, kept in typical conditions of unheated enclosed balconies and terrases: (A) May, the 1st month of the experiment. The plants are still young and have a few small leaves; the average height of the plants is 21.2 cm. (B) June, the 2nd month of the experiment. Bushes of water plantain are well formed and have 7-8 leaves, the height of the ground part is 36.1 cm on the average. (C) July, the 3rd month of the experiment. Beginning of flowering. (D) September, the 5th month of the experiment. Flowering ends, leaves begin to turn yellow. (E) October, the 6th month of the experiment. The leaves turn yellow and dry. (F) November, the 7th month of the experiment. Despite on the presence of young leaves, the plants died.

is simple and consists in watering 2-3 times per week.

So, glazed balconies and terraces – which are usually used to increase the size of the apartment by arranging rest area or workplace there, or if they are not heated, are used as a buffer zone between the apartment and the outside world to reduce the cost of heating – can perform another important, energy supply function. Glazed balcony or terrace can be seen as a greenhouse where

Table 2. The cost of components bioelectric-producing system

Elements of the biotechno-system	Materials	Cost (EUR)
Container	Polypropylene	3.27
Cathodes	Graphite plates	15.30
Anodes	Plates of galvanized steel	4.14
Conductors for connecting the cathodes	Copper multiple wires	1.19
Conductors for connecting the anodes	Copper multiple wires	1.19
Plants A. plantago-aquatica		4.70 (0.0)
Soil universal substrate		1.91 (0.0)
Water		-
Mud		-
Total		31.70 (25.09)

there is sufficient natural light, stable temperature can be maintained by underfloor or radiator heating. These areas can be used for growing *A. plantago-aquatica* with the aim of obtaining plant-microbial energy in the flats.

System cost

Our proposed electrode system for collecting bioelectricity (Fig. 1) functioned properly in wetland substrate during the 40-week experiment, and did not require replacement. The newly designed graphite-galvanized steel system of electrodes (Rusyn and Medvediev 2018) is easy in manufacture and of low cost. On average, the cost of materials for one biosystem an area of 0.0525 m^2 is 31.7 EUR as shown in Table 2.

The plants for bioelectricity generating systems can be bought in plant nurseries, as well as taken from natural reservoirs. Similarly, the soil for the system may be purchased or obtained from the natural environment. This leads to a further reduction of the cost of the system, in this case to 25.09 EUR (Table 2).

The price of materials for 1 m^2 of the system goes from 501.8 EUR (without the cost of plants and soil substrate) to 634.0 EUR (with cost of with these elements plants and soil substrate).

The cost of collecting and installing the bioelectric-system are also insignificant. The price of collecting the biotechnological system, based on the cost of the hardware for connecting the technical components of the system, at a scale 10 000 biosystems, will be inconsiderable. The installing the biological system, which is quite simple and fast.

Production of bioelectricity under different conditions

The average output voltage of the bioelectric systems

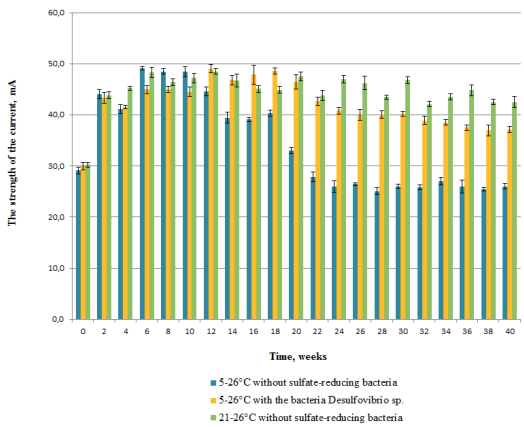

Figure 4. The average current strength of the biosystems with *A. plantago-aquatica* under 10 Ω load during 40 weeks of experiment in different conditions: at temperature fluctuations from 5 °C to 26 °C on the unheated terraces and balconies with and without sulfate-reducing bacteria and at 21 °C to 26 °C on the heated balconies and terraces without the bacteria.

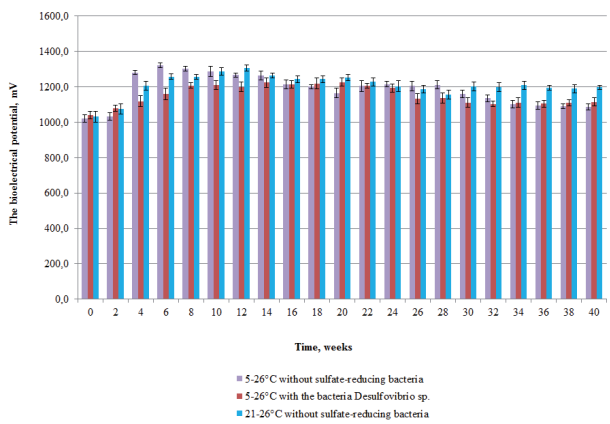

Figure 5. The average open circuit bioelectrical potential of the biosystems with *A. plantago-aquatica* during 40 weeks of experiment in different conditions: at temperature fluctuations from 5 °C to 26 °C on the unheated terraces and balconies with and without sulfate-reducing bacteria and at 21 °C to 26 °C on the heated balconies and terraces without the bacteria.

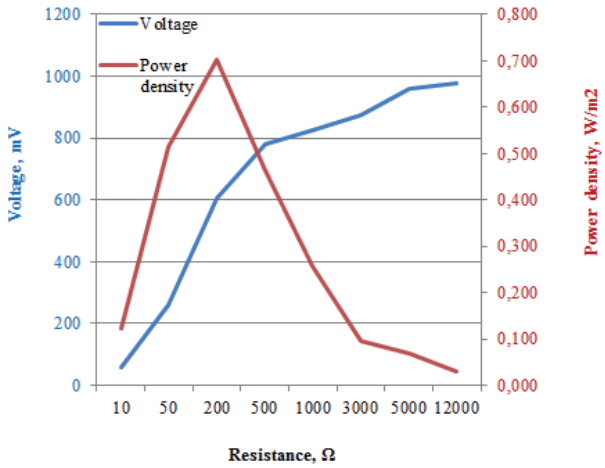

Figure 6. Output voltage and power density of bioelectrical systems with A. plantago-aquatica under the action of external resistors 10 Ω - 12 kΩ. The bioelectric potential of the electro-biosystems is close to 975 mV when using a resistor of 12 000 Ω.

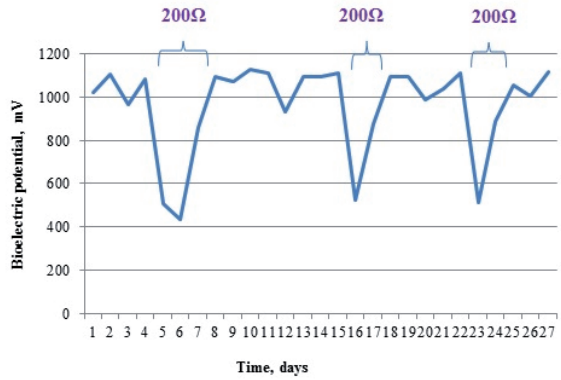

Figure 7. One-day and two-days effects of an external resistor of 200 Ω on the generation of bioelectric potential by an bioelectrical system with *A. plantago-aquatica*.

with the *Alisma plantago-aquatica* in indoor conditions of buildings during the 40-weeks of experiment ranged from 1.15 V to 1.21 V, and the average current strength was from 34.2 to 44.6 mA, depending on the conditions of cultivation (Fig. 4, 5). The maximum registered voltage was 1.34 V and current strength at an external resistance of 10 Ω was 58.6 mA.

The work of the bioelectric system under short-term action of external resistance is shown in Fig. 6. The highest power density was found at 50-500 Ω loads, in agreement with the data of Cheng et al. (2006). The maximum power density was 0.702 W/m^2 using an external resistor of 200 Ω, but long-term use of 200 Ω external load resulted decrease of output voltage by ca. 51.48% during one day and 58.27% over two or more days (Fig. 7). After to open of circuit voltage was restored during the day and returned to the initial level after 2 days (Fig. 7).

The set-up time of average annual bioelectric values in the biosystems

We propose to set up the bioelectricity generating system with seedlings of young plants (instead of growing them from seeds), so power generation starts from the first days after installation. During the first two days of our experiment, current strength increased by 67.7% and 65.3%, and amounted to 50.3mA and 48.1mA with and without addition of a suspension of sulfate reducing bacteria, respectively. The average current with 10Ω load for the second week of cultivation was 43.3 - 44.1 mA (Fig. 4). Two weeks after starting the bioelectricity generating system, the output voltage reached the annual average level (Fig. 5) and, up to that point, increased by 14.05%. So, finally, in two weeks the system operated at almost full capacity.

Dependence of bioelectric performance on the plants' development

Significant increase of power output with the development of aerial parts of plants in the system was observed. On unheated terraces and balconies, where plants undergo cyclical development,. the maximum electric output was recorded in summer and early autumn, when the bushes of plants were the most developed, contained the maximum number of developed green leaves and were photosynthetically the most active (Table. 1; Fig. 2). The highest recorded current, above 50 mA, and open circuit voltage above 1.3 V, was observed on unheated terraces and balconies in the 6th-20th weeks of experiment, in summer and early autumn. Output dropped almost to the initial level after the death of plants in systems without sulfur bacteria on unheated terraces.

Influence of external factors on the function of the system

One of the main external factors that influence on the development of plants inside buildings is temperature. In the housing stock, there are both heated glazed balconies or terraces, and closed balconies and terraces without heating where temperature drops sharply in the autumn-winter period. The biosystems are electrically productive around the year inside buildings, but the output depends on the temperature regime in which biosystems are kept. On heated terraces, where temperature is always favorable for the plants, current strength did not fall below 40 mA (Fig. 4), and was only slightly lower, by 3.88%, in the autumn-winter period. Bioelectricity generating systems on unheated balconies and terraces without the addition of sulfur bacteria were most effective only in the spring-summer period, and from the 20th week on,

with decreasing temperature and loss of plants, output was falling. Current dropped to the baseline level already in the 22th week (Fig. 4), and output voltage decreased almost to the baseline level by the 40th week (Fig. 5).

On unheated balconies and terraces, but with addition of sulfate-reducing bacteria, the time course of bioelectric power output was completely different, in spite of unfavourable temperatures. On the 26th week of the experiment, when the system with sulfate-reducing bacteria was already without living plants, average strength of the current was still 40.11 mA. Wetland substrate by itself, with the organic residues of leaf fall and with added sulfate-reducing bacteria, was obviously the source of electricity generation in these conditions. Current was maintained above 40 mA during 30 weeks of cultivation, and its subsequent decrease was insignificant (Fig. 4).

Electric power obtained from the containers with plants is the result of activity of electricity-producing microorganisms that grow by utilizing the plant root secretions (Helder et al. 2013a). Nitrogen-fixation (Beijerinck 1901) and reduction of sulfate by *Desulfovibrio* (Pfennig 1989; Moroz and Rusyn 2012) can play a key role in maximizing bioelectric output. Nitrogen supply promotes photosynthetic activity of plants, which consequently will increase the amount of nutrients excreted by the roots and utilized by the electricity producing microorganisms. Sulfate, which may be present in wetland environment in low concentration, is undesirable for the collection of bioelectricity, as it acts as an alternative electron acceptor (Ivanov et al. 2017; Morris and Jin 2009). Therefore, reduction of sulfate by *Desulfovibrio* sp., sulfate-reducing bacteria, is a positive factor in the biotechnology of bioelectricity production. And also, perhaps, sulfur-bacteria can promote the development of the power-generating bacteria by their metabolites that they secrete into the subtrate and that may directly participate in the process of producing of bioelectricity.

Although the theoretical calculated power of 3.2 W/m^2 (Strik et al. 2011) potentially available in this technology has not been achieved yet, we are still one step closer to achieving this goal using the new electrodes systems, environment and plants *A. plantago-aquatica*.

Using bioelectro-technology proposed by us, the generation of current strength is stable throughout the year at a temperature of 21 - 26 °C in well-lighted premises, glazed, heated terraces and balconies. Seasonal reduction of the bioelectricity level is equal to 8.71%. On closed balconies and terraces that are not heated, at a temperature fluctuations 5 - 26 °C, the production of bioelectricity decreases in the winter period compared with average values of the spring-summer-autumn period by 39,91% without sulfur-bacteria and by 19.98% with addition sulfur-bacteria. Such a decrease in the generation of bioelectricity in the winter period on closed terraces and balconies is insignificant compared with the same one fixed decrease of bioelectricity in green roofs in outdoor conditions of almost 5 times (Helder et al. 2013b) or with the fully termination of generation of the current of strength in wetland forests (Dai et al. 2015). This fact reveals the great potential of glazed green terraces and balconies as a source of bioelectricity in the winter period. The glazed loggias of houses, after the improvement of biotechnology, can be used to grow marsh plants *A. plantago-aquatica* in order to obtain plant-microbial energy in apartments.

Conclusions

Green gardens, located on glazed balconies or closed terraces, are an alternative to green roofs as sources of bioelectricity in countries with cold or arid climate and solve the problem of bioelectricity losses *in situ* during the cold seasons of the year. Although the power, theoretically possible in this technology, is still not achieved, the proposed bioelectro-technology with wetland plant *A. plantago-aquatica* has progress in thrift, efficiency and round-the-year collection of bioelectricity, and contains new approaches that have good prospects for further improving of the collection of plant-microbial bioelectricity.

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ARTICLE

Effect of DPP-4 inhibitor sitagliptin against ischemia-reperfusion (I/R) injury in hyperlipidemic animals

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ABSTRACT Hyperlipidemia is a major risk factor associated with increased risk of myocardial infarction. Dipeptidyl peptidase-4 (DPP-4) inhibitors such as sitagliptin are a class of oral anti-diabetic drugs with secondary pleiotropic effects on metabolic and cardiovascular parameters. This study aimed to determine the possible cardioprotective effects of sitagliptin on ischemia-reperfusion (I/R) injury in animals kept on high-fat diet. Male Wistar rats were fed with high-fat diet (HF) for 12 weeks, to induce hyperlipidemia. During the last two weeks of the feeding period, animals were orally treated with different doses of sitagliptin (Sitg: 25, 50, 100, and 150 mg/kg/day), or saline as a control. Heart tissues were then isolated and subjected to two different I/R-injury protocols for infarct size (IS) measurement and biochemical analysis. To test the role of NOS enzyme, NOS inhibitor (L-NAME) was injected intraperitoneally for IS evaluation. As an effective dose, Sitg (50 mg) exhibited a significant impact on IS. NOS activity increased significantly in the Sitg (50 mg) treated groups; however this protective effect was abolished in the presence of L-NAME. The protective effect of Sitg that was mediated by TRP channels in our previous study on normolipidemic animals was abrogated in animals fed with high-fat diet.
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KEY WORDS
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INTRODUCTION

Hyperlipidemia is considered a major risk factor for ischemic heart disease (Kupai et al. 2009) and myocardial infarction (MI) (Wu et al. 2013), due to its contribution in atherosclerotic plaque formation in coronary vessels, accompanied by increase in infarct size (IS) and cardiac apoptotic and necrotic death (Ma et al. 2013).

Experimental studies mostly addressed cardioprotective effects against ischemia-reperfusion (I/R) injury in healthy animal models, in which I/R is imposed in the absence of other diseases or risk factors such as hyperlipidemia (Kupai et al. 2009). However, studies using diseased animal models with high-fat and high-cholesterol diets showed a high susceptibility of rodent hearts to I/R injury and diminished cardioprotective outcomes (Lauzier et al. 2009). As the incidence of cardiovascular disorders with an increased risk of myocardial infarction continues to grow due to dyslipidemia and obesity, and finding new and novel therapeutic targets is still a challenge.

Most of the drugs failed to find their way into clinical practice, and no drug has been approved for infarct size limitation in patients with acute coronary syndrome (Downey and Cohen 2009). Additionally, commonly used anti-diabetic drugs against diabetes including dipeptidyl peptidase-4 (DPP-4) inhibitors, were found to be independently associated with pleiotropic effects in cardiovascular diseases (Nissen and Wolski 2007). DPP-4 inhibitors (DPP-4i) are incretin-based drugs that act by inhibiting the DPP-4 enzyme, resulting in prolonged action of glucagon-like peptide-1 (GLP-1) (Apaijai et al. 2013). In addition to their glycemic control effect, DPP-4i has been reported to exert cardioprotective actions in high-fat diet (HFD) animal models (Apaijai et al. 2013). To the best of our knowledge, only one study addressed the effect of sitagliptin on cardiovascular complications after long-term of HFD consumption (Apaijai et al. 2013), without addressing its effect against I/R injury. Consequently, the ability of these therapies to reduce the deleterious effects of high-fat diet on the cardiovascular system are still equivocal (Torekov et al. 2011).

Endothelial nitric oxide synthase (e-NOS) is constitutively expressed in cardiomyocytes, while hypercholesterolemia was found to be associated with impaired endothelial function in coronary circulation (Kuo et

al. 1992), lower nitric oxide (NO) production (White et al. 1994). Moreover, reduced e-NOS expression was observed in heart tissues of hypercholesterolemic rabbit model (Onody et al. 2003). The correlation between DPP-4 inhibitors and nitric oxide synthase (NOS) system was depicted in few studies; however, the potential role of NOS remains unclear in ischemic heart. Transient receptor potential (TRP) channels including the Canonical (TRPC) and Vanilloid (TRPV) subfamilies are non-selective calcium (Ca^{2+}) permeable ion channels (Nilius and Droogmans 2001) vastly expressed in sensory nerve fibers that innervates the heart, blood vessels and vascular endothelial cells (Shenton and Pyner 2014). These channels became the subject of growing research interest over the last decades, due to their disparate effects in cardiovascular complications, but their protective effects against myocardial I/R injury were scarcely reported (Vemula et al. 2014).

Whether sitagliptin-induced cardioprotection against I/R injury can be mediated by NOS and/or TRP channels in hyperlipidemia is still unknown. Therefore, in this study we aimed to elucidate the possible protective mechanisms of sitagliptin against I/R injury and myocardial infarct size (IS) in a hyperlipidemic rat model, in comparison with the results from normolipidemic animals in our previous study (Al-Awar et al. 2018).

We have previously tested the hypothesis in normolipidemic animals (Al-Awar et al. 2018), and we found that NOS and transient receptor potential (TRP) channels are potent mediators in sitagliptin-induced cardioprotection.

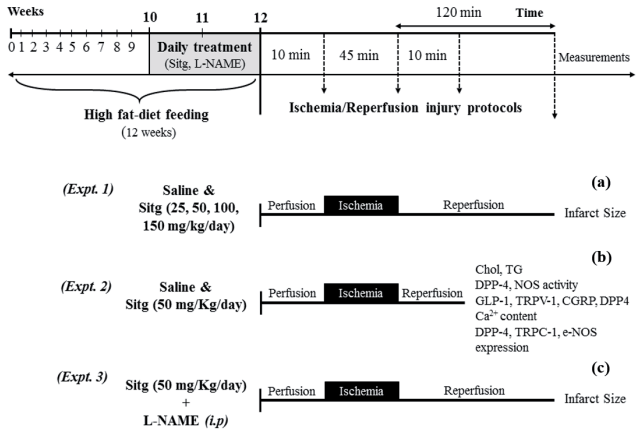

Figure 1. Summary of 3 different experimental protocols. (a) Heart tissues subjected to 45 min ischemia and 120 min of reperfusion, after two weeks of oral treatment with saline and different doses of Sitg, for infarct size measurements. (b) Hearts subjected to 45 min ischemia and 10 min brief reperfusion for biochemical measurements. (c) Infarct size measurement from heart tissues exposed to prolonged ischemia-reperfusion injury, after 2-weeks co-treatment with saline or Sitg (50 mg), and intraperitoneal injection of NOS-inhibitor (L-NAME).

MATERIALS AND METHODS

Drug preparations

Sitagliptin filmtablets (Januvia 100 mg, Merck, Hertfordshire, UK) were purchased and dissolved in physiological saline solution (0.9%) before each oral treatment and according to different intended doses. Tiopental (Tiobarbital Braun, 0.5 g, B. Braun Medical USA) was dissolved in saline (0.9%) and used as anesthetic agent. NOS-inhibitor (Nω-nitro-L-arginine methyl ester hydrochloride; L-NAME), purchased from Sigma-Aldrich and dissolved in physiological saline (0.9%).

Animals and experimental design

Our study falls within the standards of the European Community guidelines for the Care and Use of Laboratory Animals. All procedures were performed according to the protocols approved by the Institutional Ethical Animal Care and Use Committee of University of Szeged, with the project identification code and date of approval (XX/4801/2015, 15 December 2015). Six to eight-week-old male Wistar rats (body weight 200-300 g; Toxi-Coop, Dunakeszi, Hungary) were obtained and acclimatized for one week before the onset of treatments. All animals were housed in temperature-controlled animal facility (23 °C) that belongs to our lab and maintained with a 12h-12 h light-dark cycle with food and water provided *ad libitum*. Animals were fed with standard rat chow mixed with fats (High fat = HF) for 12 weeks to induce hyperlipidemia.

Animals were assigned into 3 different experiments:

Experiment 1. To determine the effective dose ($kg^{-1}day$) of sitagliptin (Sitg), animals were randomly divided into different groups: (Control (saline), Sitg (25 mg), Sitg (50 mg), Sitg (100 mg) and Sitg (150 mg)/kg/day; n = 5-10). Daily oral treatment with the different doses of Sitg or its vehicle (Saline) started at the last two weeks of the high fat diet (2.5%) feeding. At the end of the treatment, the whole-heart preparation and ischemia-reperfusion (I/R) injury protocol was performed. Rats were anesthetized with thiopental (i.p. 100 mg/kg), heart tissues were rapidly excised, placed in ice-cold saline (0.9%), mounted and ligated through the aorta into the cannula (*ex vivo*) of a modified Langendorff Apparatus, and perfused with Krebs buffer (118 mM NaCl, 4.70 mM KCl, 2.50 mM $CaCl_2$, 1.18 mM $MgSO_4$, 1.18 mM KH_2PO_4, 5.50 mM glucose and 25 mM $NaHCO_3$) than gassed with 95% O_2 and 5% CO at 37 °C. Hearts were exposed to 10 min perfusion, 45 min prolonged regional ischemia by the occlusion of the left anterior descending (LAD) coronary artery, and 120 min reperfusion. At the end of reperfusion, the LAD

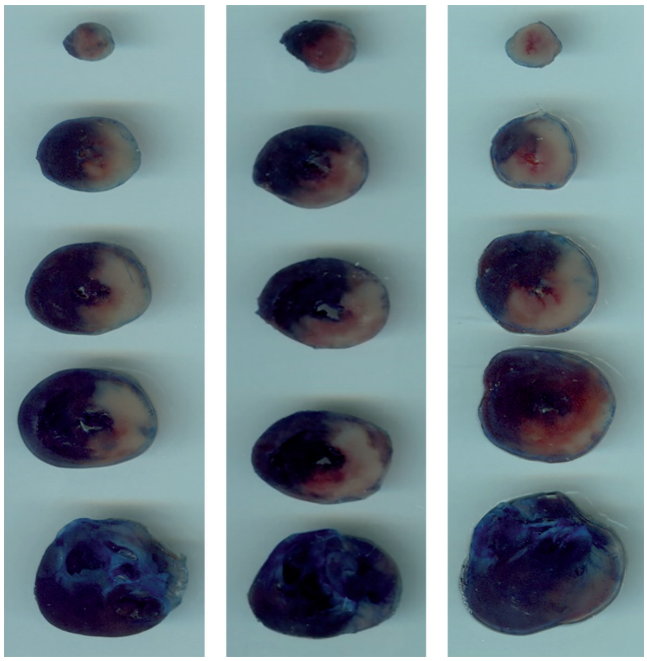

Figure 2. Representative photographs of transversely-sectioned, Evans-blue perfused and TTC-stained heart tissues outlining the area at risk (AAR; sum of white and red area); blue, healthy viable tissue; and pale white, infarcted tissue. Myocardial infarct area (IS; white) was measured post-myocardial ischemia-reperfusion and TTC staining, in treated and control groups.

coronary artery was religated, and the area at risk (AAR) was stained with Evans blue dye via the aortic root. Hearts were weighed and stored at -20 °C for further staining with triphenyltetrazolium chloride (TTC). In the clinical setting, the importance lies in the dose- protective effect of any drug; therefore, we considered the 50 mg dose of sitagliptin as the effective dose for biochemical measurements (Fig. 1a).

Experiment 2. For *in vitro* laboratory measurements, another set of experiments was carried out, by assigning only two animal groups (Control (Saline) and Sitg (50 mg), n = 10). At the end of the treatment, same anesthetization procedure and whole-heart preparation process were carried out as in *Experiment 1.* Cannulated hearts were exposed to 10 min perfusion, 45 min prolonged regional ischemia by occluding the LAD coronary artery, followed by 10 min brief reperfusion. At the end of the experiment, heart tissues were weighed, clamped and stored at -80 °C for further biochemical analyses (Fig. 1b).

Experiment 3. To confirm the involvement of NOS in Sitg-mediated (50 mg) cardioprotection against IR injury, four different animal groups (Control (Saline), Sitg (50 mg), Control (Saline) + L-NAME, and Sitg (50 mg) + L-NAME, n = 6-8) were included. The Control (Saline) and Sitg (50 mg) animal groups received the same daily oral treatment as in *Experiment 1,* while the other two groups were co-treated intraperitoneally (i.p.) with the NOS inhibitor (L-NAME, 25 mg/kg/day) (Jaarin et al. 2015), three hours' post-oral administration of Sitg (50 mg) and its vehicle (saline). At the end of the treatment, the same anesthetization procedure, whole-heart preparation process, and I/R injury protocol (10 min perfusion, 45 min prolonged regional ischemia and 120 min reperfusion, *ex vivo*), coronary artery re-ligation, and tissue staining procedure were carried out as in *Experiment 1* (Fig. 1c).

Tissue staining and infarct size (IS) measurements

At the end of each prolonged reperfusion phase (120 min), the LAD coronary artery was re-ligated, and the risk zone was stained with Evans blue dye via the aortic root. Hearts were frozen, transversely sectioned (5-6 slices, 2-mm thickness) from the apex to the base, and incubated in 1% TTC for 10 min at 37 °C. After incubation, tissue sections were fixed for 10 min in 10% formalin and placed for 30 min in phosphate buffer (pH 7.4). All sections were mounted on glass slides, images were captured with a digital camera, and infarcted areas were analyzed using an ImageJ 1.34 software. The infarction was analyzed in each section by an investigator who was blinded to the identity of the sections (Fig. 2).

Serum cholesterol and triglyceride measurements

After removal of heart tissues, blood samples were taken from the abdominal aorta, centrifuged, and serum samples were collected in Eppendorf tubes and stored at -20 °C for cholesterol (Chol) and triglyceride (TG) measurements. Chol and TG reagent kits (Diagnosticum, Hungary) were used for both measurements. Quantitative determination of cholesterol and triglyceride concentration in serum was based on enzymatic colorimetric method (PAP). Standard and sample (10 µl) measurements at wavelength 490-550 nm were carried out in 96-well plates, after 5 min incubation at 37 °C and according to the protocols provided in the kit's manual. Results of both measurements were expressed in (mmol/l).

Hepatic cholesterol and triglyceride measurements

At the end of the treatment and after the animals were anesthetized, liver tissues were harvested, rapidly clamped in liquid nitrogen and stored at -80 °C. Measured samples from liver were homogenized in ice-cold modified phosphate buffer saline (PBS) by Ultra-Turrax T25 (13 500 rpm). Liver supernatants were collected, and the same Chol- and TG-kits (Diagnosticum, Hungary) were used with some modifications in the provided protocols regarding dilutions and sample volume. Obtained results were expressed in mmol/l.

DPP-4 activity test

Cardiac DPP-4 activity was measured in Control (Saline) and Sitg (50 mg) treated groups, using a DPP-4 activity assay kit and according to the manufacturer's guidelines (Sigma-Aldrich, St. Louis, USA). Ten mg of heart tissue were homogenized in ice-cold DPP-4 Assay Buffer, centrifuged at 13 000 g for 10 min (4 °C), after which supernatants were collected. Standard and sample fluorescence intensity (FLU) measurements (λ_{ex} = 360 nm / λ_{em} = 460 nm) were carried out after 5-min incubation at (37 °C) in 96-well black plates designed for fluorescence assays, using a fluorescence multi-well plate reader. Incubation and measuring cycles were repeated until the most active sample was near to or greater than the value of the highest standard (100 pmol/well). Results were expressed as microunit/ml.

Nitric oxide synthase (NOS) activity

NOS activity was measured by quantifying the conversion of [^{14}C]-labeled L-arginine to citrulline by a previously described method with some minor modifications (Boughton-Smith et al. 1993). Heart tissues were homogenized with Ultra-Turrax T25 (13 500 rpm; twice for 30 sec) in ice-cold buffer containing 10 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), 32 mM sucrose, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 2 μg/ml aprotinin, at pH 7.4 (all chemicals were from Sigma-Aldrich, Budapest, Hungary). Supernatants were collected after centrifugation (20 000 g, 30 min, 4 °C). Samples (40 μl) were incubated for 10 min at 37 °C with 100 μl of assay buffer (50 mM KH_2PO_4, 1.0 mM $MgCl_2$, 50 mM L-valine, 0.2 mM $CaCl_2$, 1.0 mM dithiotreitol (DTT), 1.0 mM L-citrulline, 15.5 nM L-arginine, 30 μM flavin adenine dinucleotide, 30 μM flavin mononucleotide, 30 μM tetrahydro-L-biopterin dihydrochloride, 450 μM β-nicotinamide adenine dinucleotide phosphate (β-NADPH), and 12 pM [^{14}C]-L-arginine monohydrochloride (all chemicals were from Sigma-Aldrich). Reactions were terminated by the addition of 0.5 ml of a 1:1 (v/v) suspension of ice-cold DOWEX (Dowex 50WX8 hydrogen form 100-200 mesh, Sigma Aldrich) prepared in distilled water. The mixture was resuspended by adding 850 μl of ice-cold distilled water, and 970 μl of supernatant was removed for determination of radioactivity by scintillation counting. Ca^{2+} dependence of NOS activity was determined by adding 10 μl of ethylene glycol-bis (β-aminoethyl ether) tetraacetic acid (EGTA; 1 mM, Sigma-Aldrich). NOS activity was confirmed by inhibition with 10 μl of Nω-nitro-L-arginine methyl ester (L-NNA; 3.7 mM, Sigma-Aldrich). The level of inducible NOS (i-NOS) was defined as the extent of citrulline formation that was inhibited by L-NNA, but not by EGTA. Constitutive NOS (cNOS) activity was calculated from the difference between the extent of citrulline formation inhibited by EGTA and the total activity. As the nature of the cNOS isoform (e-NOS or n-NOS) was not determined, this activity is referred to as cNOS. NOS activity was expressed as pmol/min/mg protein.

ELISA measurements (GLP-1, TRPV-1 and CGRP)

Double-antibody sandwich ELISA kits for rat GLP-1, TRPV-1 and CGRP measurements were purchased from SunRed Biotechnology (Shanghai, China). The same homogenization procedure (Ultra-Turrax T8, 20 min centrifugation at 2000-3000 rpm) was followed, using the PBS homogenization buffer (pH 7.2-7.4). Tissue sample preparation procedure was done on ice. The three parameters were measured according to the manufacturer's protocols, and optical densities (OD) were determined at 450 nm wavelength. Results are expressed in ng/ml for GLP-1 and TRPV-1 and ng/mg protein for CGRP.

Calcium (Ca^{2+}) content test

A colorimetric Calcium Detection Assay Kit (Abcam, Cambridge, UK) was used to determine the calcium (Ca^{2+}) concentration. Samples were homogenized on ice using PBS + 0.1% NP-40 and centrifuged at a maximum speed (15 300 rpm) for 2-5 min at 4 °C. Measurements from supernatants were performed according to the provided procedure. OD were measured (λ = 575 nm) and results were expressed in ng/mg protein.

CD26 (DPP-4), TRPC-1 and e-NOS protein expression by Western blotting normalized to β-actin

Measured heart tissues were homogenized by Ultra-Turrax T25 (13 500/s; twice for 30 sec) with ice-cold radio immunoprecipitation assay (RIPA) buffer (containing a protease inhibitor and TRITON-X-100), for CD26 (DPP-4) and TRPC-1 proteins, and Homo-buffer (containing phosphatase inhibitor, vanadate (1:50)), for e-NOS. Homogenates were centrifuged (12 000 rpm, 10-15 min, 4 °C). Proteins were separated by an 8% and 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE, 1 mm gel cassette), and transferred into nitrocellulose membranes. Blots were probed overnight (4 °C, and 1% milk) with anti-TRPC-1 rabbit primary antibody (1:500, ab192031, Abcam), and anti- eNOS mouse primary antibody (1:250, ab 76198, Abcam), respectively, 2 hours at room temperature with anti-CD26 rabbit primary antibody (1:500, ab129060, Abcam), and anti-beta actin mouse primary antibody (1% BSA, 1:4000, ab 8226. Abcam). Membranes were then incubated for 1 h at room temperature with secondary anti-rabbit antibody (1:1000, sc-2370, Santa Cruz, TX, USA), secondary anti-mouse an-

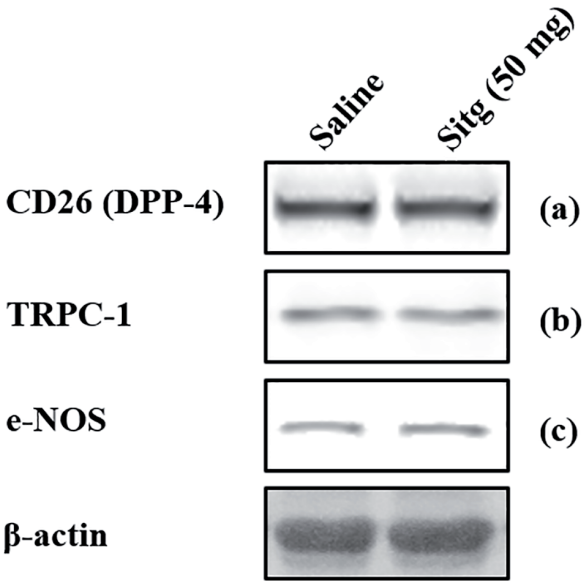

Figure 3. Protein expression of CD26 (DPP-4), TRPC-1 and e-NOS (a, b and c, respectively) in heart tissues from Sitg-treated (50 mg) group, compared to the Control (saline) group.

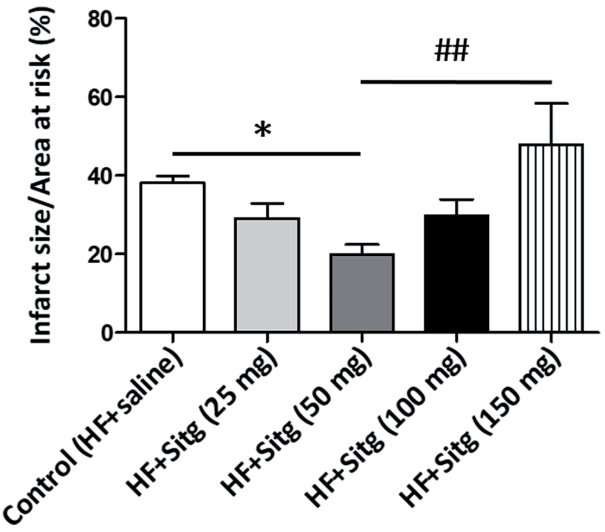

Figure 4. Effect of different doses of Sitg on infarct size (expressed in %). Results are shown as Mean ± SEM; n = 5-10 animals/group. Statistical significance is represented as: $^{*}P < 0.05$ compared to the Control group, and $^{\#\#}P < 0.01$ comparing HF + Sitg (50 mg) and HF + Sitg (150 mg) groups together. Sitg (50 mg) exhibited a significant decrease in infarct size, while no significance was observed in other doses (25, 100, and 150 mg).

tibody (1:5000, A9044, Santa Cruz), secondary anti-rabbit (1:5000, sc-2370, Santa Cruz), and secondary anti-mouse antibody (1:2000, A9044, Santa Cruz) for TRPC-1, e-NOS, CD26 and β-actin, respectively. Secondary antibodies were conjugated with horseradish peroxidase (HRP) enzyme. Signals were developed using an enhanced chemiluminescent substrate for detection of HRP (ECL Western Blotting Substrate, Thermo Scientific, Rockford, USA) and exposed to Hyperfilm. Films and protein band densities were analyzed using the Image Quant Software (Amersham Pharmacia, Buckinghamshire, UK) after scanning with Gel Analyst 3.01 Software (Iconix, Toronto, Canada), and were normalized to housekeeping protein β-actin (Fig. 3).

Protein determination

Aliquots (20 µl) from diluted samples (15- or 25-fold with distilled water) were mixed with 980 µl of distilled water and 200 µl of Bradford reagent was added to each sample. After mixing and 10-min of incubation, samples were assayed spectrophotometrically in a microplate reader at 595 nm with a commercial protein assay kit (Bio-Rad, Budapest, Hungary). Protein levels were expressed as mg protein/ml.

Statistical analysis

All data are shown as Mean ± standard error of mean (SEM). Statistical comparisons were performed with Student's two-tailed unpaired t test, one-way ANOVA multiple comparison test (Bonferroni), and two-way ANOVA, when necessary. Differences were considered significant when $P< 0.05$.

RESULTS

DPP-4i decreased infarct size (IS) in heart tissues of Sitg (50 mg) group

Two weeks following the daily oral administration of different doses of the same DPP-4 inhibitor (Sitg), Sitg treatment (50 mg/kg) resulted in a significant decrease in IS (19.99 ± 2.44%) compared to the control group (38.11 ± 1.82%), after 45 min of regional ischemia and 120 min of reperfusion. The area of infarction is expressed as the percentage of infarct size over the area at risk (Fig. 4).

Serum cholesterol and triglycerides concentration

Cholesterol measurements from serum samples revealed significant increase in high-fat diet animals (HF + saline; 2.72 ± 0.15 mmol/l) compared to normal control animals (Saline; 1.95 ± 0.17 mmol/l). Sitagliptin administration (HF + Sitg (50 mg)) failed to reduce cholesterol levels (2.75 ± 0.10 mmol/l) when compared to control animal group kept on high-fat diet (HF + saline; 2.72 ± 0.15 mmol/l). Results are shown in Fig. 5a.

Similarly, serum triglyceride level was significantly increased in HF + saline vs. Control (saline); 2.12 ± 0.13

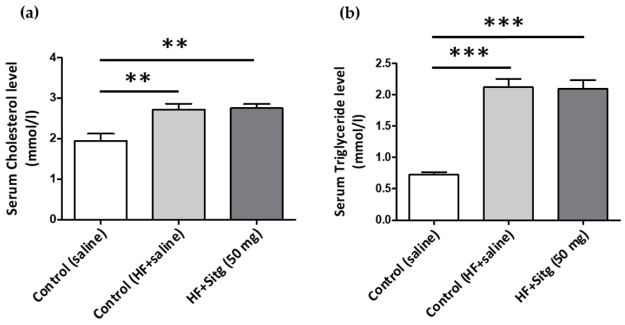

Figure 5. Serum cholesterol (a) and triglyceride (b) levels (expressed in mmol/l) in control animals kept on normal or high-fat diet, and high-fat diet animals treated with sitagliptin. Data are presented as Mean ± SEM; n = 5-8 animals/group. One-way ANOVA multiple comparison test (Bonferroni) was used for statistical analysis, and results were considered significant when $^{*}P < 0.05$.

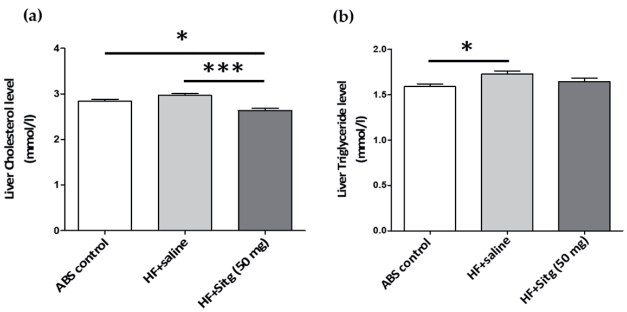

Figure 6. Liver cholesterol (a) and triglyceride (b) levels expressed in mmol/l. Results are considered significant when $^{*}P < 0.05$. Data are presented as Mean ± SEM (n = 4-5 animals/group).

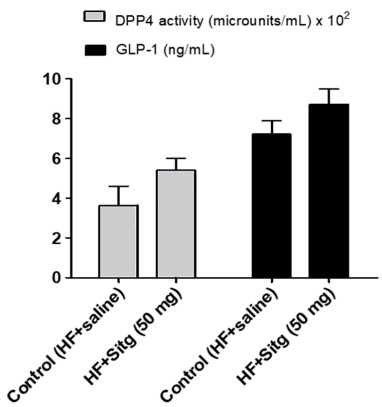

Figure 7. Ischemic cardiac tissue GLP-1 level and DPP-4 activity after two weeks oral treatment with Sitg (50 mg). Results are expressed in ng/ml and microunits/ml x 10^2, for GLP-1 and DPP-4 activity, respectively. Presented data are presented as Mean ± SEM (n = 4-10 animals/group).

vs. 0.72 ± 0.04 mmol/l. In addition, no significant decrease was observed in triglyceride level in sitagliptin-treated group kept on high-fat diet (HF + Sitg (50 mg); 2.09 ± 0.14 mmol/l) (Fig. 5b).

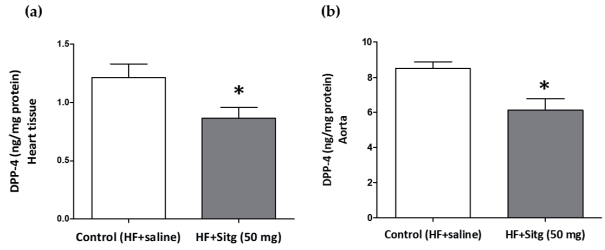

Figure 8. Changes in DPP-4 level (expressed in ng/mg protein) in heart tissues (a) and aorta (b) of Sitg (50 mg) treated animal group (HF + Sitg (50 mg)), compared to Control (HF + saline). Data are presented as Mean ± SEM (n = 5-7 animals/group). Statistical significance: $^{*}P < 0.05$ compared to the control group.

Serum cholesterol and triglyceride concentration

Measurements from liver homogenates showed a significant decrease in cholesterol level in HF + Sitg (50 mg) group compared to both the HF + saline group (2.64 ± 0.03 vs. 2.94 ± 0.04 mmol/l), and the absolute (ABS) control group (2.64 ± 0.03 vs. 2.86 ± 0.04), respectively. However, no change in cholesterol profile was reported when comparing the HF + saline group (2.924 ± 0.044 mmol/l) to the ABS control group (2.982 ± 0.146 mmol/l) (Fig. 6a). On the contrary, liver triglyceride exhibited a significant increase in animals kept on high-fat diet (HF + saline; 1.73 ± 0.03 mmol/l), compared to the absolute control group (ABS control; 1.59 ± 0.03 mmol/l). On the other hand, no significant difference in hepatic triglyceride was observed in high-fat diet group treated with sitagliptin (HF + Sitg (50 mg)) compared to high-fat diet control group (HF + saline), (1.65 ± 0.04 mmol/l vs. 1.73 ± 0.03 mmol/l) (Fig. 6b).

Effect of Sitg on heart tissue DPP-4 activity and GLP-1 level

Measurements from heart tissues subjected to brief reperfusion (10 min) showed no significant change in GLP-1 levels (8.72 ± 0.76 ng/ml vs. 7.21 ± 0.67 ng/ml) and DPP-4 activity (5.41 ± 0.95 microunits/ml x 10^2 vs. 3.64 ± 0.95 x 10^2), in sitagliptin-treated groups compared to Controls (Fig. 7).

Sitg (50 mg) normalized high DPP-4 level in heart tissues and aortas of control group

Heart tissues (Fig. 8a) and aortas (Fig. 8b) exhibited a significant reduction in DPP-4 level (0.87 ± 0.09 vs. 1.22 ± 0.12 ng/mg protein) and (6.13 ± 0.55 vs. 8.52 ± 0.34 ng/mg protein), respectively, in Sitg (50 mg) group vs. Control (saline).

Effect of DPP-4i treatment on DPP-4 protein expression

No significant difference in DPP-4 (CD26) protein expression was noticed in HF + Sitg (50 mg) group (554.17

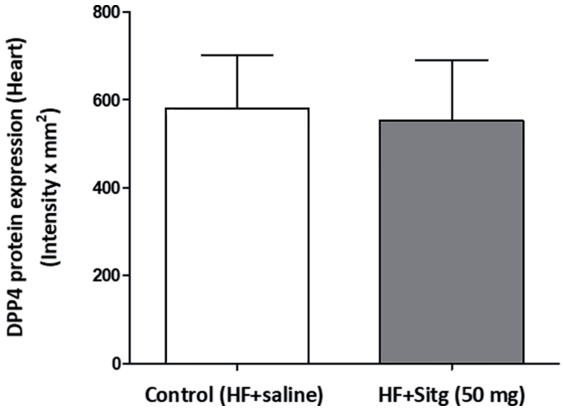

Figure 9. DPP-4 protein expression level (expressed in intensity x mm^2) in heart tissues of HF + Sitg (50 mg) group (n = 6), compared to control group (HF + Saline; n = 5) group. Data are presented in term of Mean ± SEM.

± 136.91 intensity x mm^2) vs. HF + Control (saline) group (581.03 ± 121.71 intensity x mm^2), after normalization to β-actin (Fig. 3a and 9).

DPP-4i increased CGRP but not TRPV-1 levels

No marked change in heart TRPV-1 level was observed in Sitg-treated (50 mg) group (4.08 ± 0.28 ng/ml x 10) compared to the Control (5.23 ± 0.31 ng/ml x 10). CGRP level was augmented in Sitg (50 mg) group (16.65 ± 1.04 ng/mg protein) vs. Control group (10.93 ± 1.84 ng/mg protein) (Fig. 10).

Enhanced cardiac calcium (Ca^{2+}) content in Sitg-treated (50 mg) group

To determine whether sitagliptin impacts calcium concentration of the ischemic myocardium in hyperlipidemic state, a colorimetric calcium detection assay was

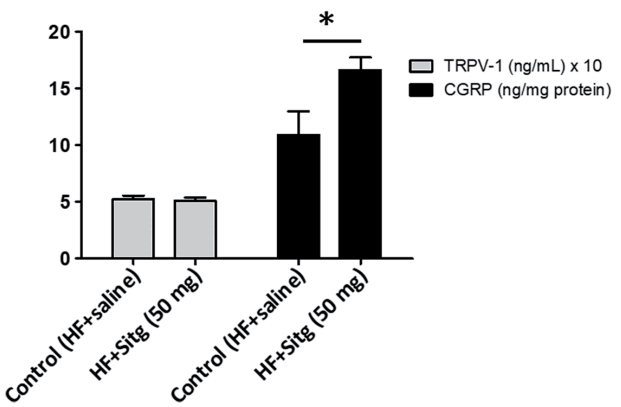

Figure 10. Effect of 50 mg dose of Sitg on cardiac TRPV-1 and CGRP levels, after two weeks oral treatment. Data are illustrated as Mean ± SEM; (n= 4-9 animals/group).

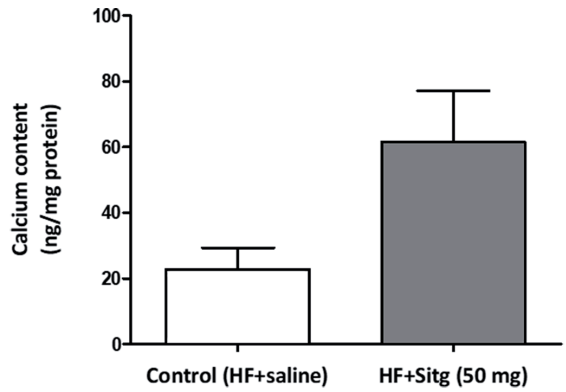

Figure 11. Changes in cardiac calcium content of the Sitg (50 mg) treated animals, compared to the control ones. Values are in terms of Mean ± SEM.

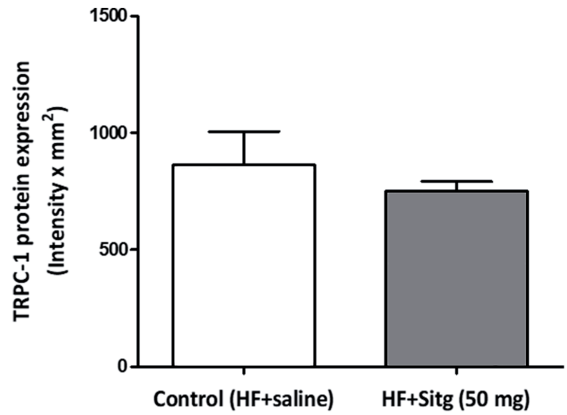

Figure 12. Unaffected TRPC-1 protein expression level (intensity x mm2) in heart tissues of HF + Sitg (50 mg) treated group (n = 8) vs. Control group (HF + saline; n = 8). Data are presented as Mean ± SEM.

used. Results showed non-significant change in calcium content in heart tissues subjected to drug therapy (61.52 ± 13.51 ng/mg protein) vs. Controls (22.79 ± 6.53 ng/mg protein) (Fig. 11).

TRPC-1 protein expression level

The difference in TRPC-1 protein expression level between the Control (HF + saline) and HF + Sitg (50 mg) treated groups is presented in Figures 12 and 3b. Unlike the results from normolipidemic animals (Al-Awar et al. 2018), Sitg-treated (50 mg) group exhibited a slight non-significant decrease (752.19 ± 40.11 intensity x mm^2) in TRPC-1 expression in comparison with the Control group (862.77 ± 143.44 intensity x mm^2).

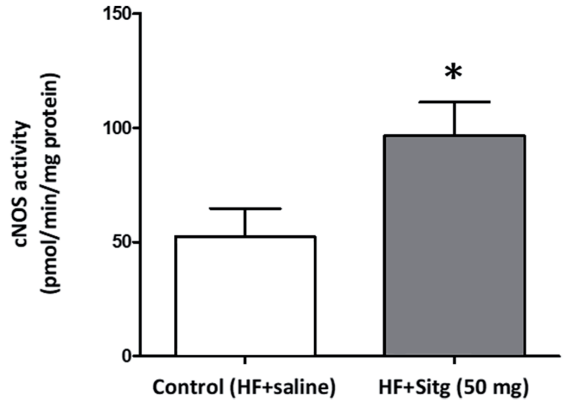

Figure 13. Significant increase (*P < 0.05) in constitutive nitric oxide synthase (cNOS) activity in heart tissues of HF + Sitg (50 mg) group (n = 7), compared to Control group (HF + saline; n = 7). Values are expressed in pmol/min/mg protein. Data are presented as Mean ± SEM.

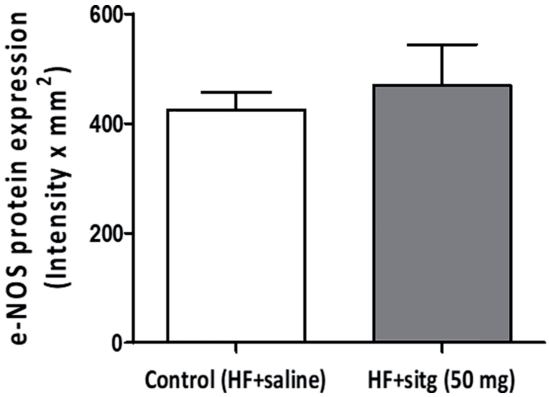

Figure 14. Unaffected level of e-NOS expression (expressed in Intensity x mm^2) in heart tissues of HF + Sitg (50 mg) group (n = 8), compared to Control group (HF + saline; n = 6). Data are presented as Mean ± SEM.

DPP4-i upregulated cNOS activity and e-NOS protein expression in heart tissues of Sitg (50 mg)

cNOS activity

Two weeks of daily treatment with Sitg (50 mg), followed by excision of heart tissues and brief reperfusion (45 min occlusion and 10 min reperfusion) of the coronary artery, lead to a significant increase in cNOS activity (96.51 ± 13.75 pmol/min/mg protein), compared to the Control group (52.38 ± 11.56 pmol/min/mg protein) (Fig. 13).

e-NOS protein expression

Expression of endothelial nitric oxide synthase (e-NOS) as determined by Western blot is shown in Figures 3c and 14. The level of expression was insignificantly changed in HF + Sitg (50 mg) treated animals (470.32 ± 73.79 intensity x mm^2), compared to the Controls (425.26 ± 31.77 intensity x mm^2).

Effect of L-NAME on NOS-mediated cardioprotection against infarct size

The implication of NOS in DPP-4 inhibition-induced cardioprotection was confirmed by treating the animals with NOS-inhibitor (L-NAME) and measuring the size of infarction. The current results are in line with the previous work in normolipidemic animals, showing a significant reduction (18.47 ± 1.22%) in Sitg (50 mg) group, compared to Control (HF + saline) group (43.33 ± 1.86%). However, this protective effect was lost in HF + Sitg (50 mg) + L-NAME group (59.17 ± 6.09%) when compared to the results obtained from animals treated with Sitg (50 mg) without L-NAME (HF + Sitg (50 mg); 18.47 ± 1.22%) (Fig. 15).

DISCUSSION

DPP-4 inhibitors were extensively studied in healthy animal models as a remedy for cardiovascular disorders; however, the interventional mechanisms of the latter drugs were poorly addressed in diseased models, such as hyperlipidemia. This study was carried out with the aim of extending the results of previous work in normolipidemic animals (Al-Awar et al. 2018). In this HF rat model, the effect of hyperlipidemia on the development of myocardial infarction (MI) following a temporary coronary occlusion (ischemia-reperfusion injury) was studied. We hypothesized that hypercholesterolemia can be associated with increased infarct size, and sitagliptin can decrease its detrimental effect on the heart, clarifying mechanisms underlying this protection.

Treatment with Sitg (50 mg) showed a significant decrease in infarct size and increase in cNOS activity in comparison with the control group, while this infarct size- limiting effect was abolished after NOS-inhibition by L-NAME, similarly as in normolipidemic animals (Al-Awar et al. 2018). L-NAME is a non-selective NOS inhibitor that inhibits the 3 NOS isoforms: endothelial NOS (e-NOS), inducible NOS (i-NOS) and neuronal NOS (n-NOS). According to a previous study, NOS inhibition also blocked its protective effect against myocardial infarct size in high-fat diet-fed animal model (Ding et al. 2015).

Sitagliptin exhibited a lowering effect in liver cholesterol, but not in liver triglycerides. Moreover, no significant change was observed in serum Chol and TG levels in animal groups treated with Sitg (50 mg), compared to the controls. According to previous clinical studies, treatment with 50 mg dose of sitagliptin was effective in lowering lipid profile and glucose levels, and reducing DPP-4 activity by approximately 80% in patients with type 2 diabetes (Herman et al. 2005; Shigematsu et al. 2014)

In the contrary of obtained findings from normolipidemic animals (Al-Awar et al. 2018), the expected decrease in DPP-4 activity and increase in GLP-1 was not observed in high-fat diet condition. However, treatment with sitagliptin exhibited a significant decrease in DPP-4 protein level measured from heart tissues and aortas. Taking into consideration the dietary factor alone, a significant increase in GLP-1 level can be observed in hyperlipidemic (Control (HF + saline)) group compared to the normolipidemic (Control (Saline)) group, making high-fat diet a suspicious factor in blocking the protective effect of sitagliptin.

On the level of protein expression, results revealed no remarkable change in CD26, e-NOS and TRPC-1 protein levels, comparing the Sitg-treated groups to Control ones. On the other hand, a significant increase in e-NOS and TRPC-1 expression was observed in animals kept on normal diet and treated with sitagliptin (Al-Awar et al. 2018).

It was previously suggested that hypercholesterolemia can partially block the ion channels and its membrane receptor downstream signaling by reducing membrane fluidity, leading to cardiomyocyte dysfunction (Wu et al. 2017). This blockage might be also evident in the current study, because the protective effect of Sitg shown to be mediated by TRPV/TRPC upregulation in normolipidemic animals, was lost after a long-term consumption of high fat- diet. Obtained results from CGRP measurement are in line with the results obtained from animals fed with standard diet (Al-Awar et al. 2018). This suggests that Sitg can have a direct effect on CGRP in hyperlipidemic condition, independently from TRP channels.

Based on the obtained results of infarct size analysis, we extended our experiments to study the mechanisms and biochemical markers underlying the Sitg (50 mg) dose. We studied the effect of sitagliptin in an *ex vivo* I/R injury model. However, investigating the same drug using an *in vivo* I/R injury model can support the protective effects and clinical relevance of sitagliptin.

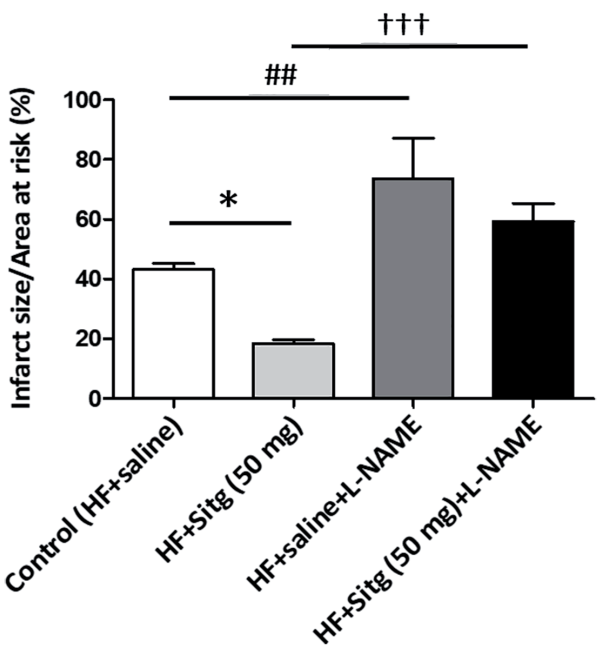

Figure 15. Loss of Sitg-induced (50 mg) cardioprotection mediated by NOS, translated by increase in infarct size (%), after intraperitoneal injection of NOS inhibitor (L-NAME). Statistical analysis was performed using One-way ANOVA, as well as two-way ANOVA when necessary. Data plotted as Mean ± SEM.

Conclusions

Sitagliptin was effective in lowering the infarct size (IS) and upregulating nitric oxide synthase (NOS). However, its protective effect mediated by transient receptor potential (TRP) channels in normolipidemic animals was abolished under hyperlipidemic condition.

Acknowledgements

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ARTICLE

Seasonal variation in prevalence, parasite load and mean intensity of ectoparasites in *Pipistrellus kuhlii* (Chiroptera: Vespertilionidae) from Iran

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ABSTRACT We studied quantitatively the seasonal variation in prevalence, parasite load, and mean intensity of two species of ectoparasites in free ranging populations of Kuhl's bat, *Pipistrellus kuhlii* in western Iran. In total, 348 live bats (230 males and 118 females) were collected using mist net during May to November 2013. All bats identified as to sex and the number of ectoparasite species in each bat was counted. On 348 *P. kuhlii* captured, 5355 ectoparasites were observed. Two species of ectoparasites belonging to *Argas vespertilionis* (Argasidae) and *Steatonyssus* sp. (Macronyssidae) were identified. In late May and at the beginning of June, the peaks of ectoparasite load in bats were recorded and then dropped during June to November. The number of ectoparasites in pregnant female bats in May-June during pregnancy was significantly higher than non-pregnant females ($p \leq 0.05$). A significant correlation was found between ectoparasite load and the ratio of body mass to the length of forearm (W/FA), as an index of body condition, indicating that parasite load has apparent impact on bat's health. Our findings indicated that parasite loads correlate with season, sex and reproductive condition of the host.
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Introduction

Ectoparasites live and feed on external surfaces of its host and are present on almost all species of mammals, including bats. Bats are one of the most diverse mammals and harbor numerous ectoparasites (Moura et al. 2003) which are themselves parts of the biodiversity. They are, also, dependent on the host to complete their own life-cycle (Sharifi et al. 2008, 2013). Studies on various groups of vertebrates have shown that ectoparasites influence host fitness (Fenner and Bull 2008), body condition and reproductive success (Neuhaus 2003), as they can cause mortality, morbidity, reduce fecundity or regulate host population size and change demographic characteristics (Tomás et al. 2007). Therefore, it is important to understand ectoparasites species richness, abundance, and life history in order to recognize the potential impact on host fitness (Lourenço and Palmeirim 2007). Species richness and diversity of bat ectoparasite assemblages may be related to several factors including, home range, behavior, size and roost type of the host (Zhang et al. 2010; Webber et al. 2015).

Studies on host-parasite relationships in free living bat species shows that ectoparasitic load appears to vary among male and female hosts. Most studies found that males are more parasitized than females (Morand et al. 2004, Amo et al. 2005). In contrast, study on five species of bats showed that all ectoparasites were more prevalent on female than male hosts (Christe et al. 2007). This may be due to the congregation of hundreds of individuals in many species of female bats in maternity colonies that despite thermoregulatory benefits, adult females and young may face high rates of ectoparasite exposure (Czenze and Broders 2011). There are several important biological differences between males and females that may explain sex biased parasite load. Differences in sex-specific behaviors such as aggressive behavior between males for mating may increase the likelihood of becoming more infected and cause different exposure to parasites (Krasnov et al. 2005). Also, negative association between steroid hormones, particularly testosterone, and immune responses are thought to play a major role in the difference between male and female ectoparasite load (Roberts et al. 2004)

There are studies that have reported the relationship between the number of parasites and the condition of bat health (Christe et al. 2000; Sharifi et al. 2008, 2013). In some bats, ectoparasites reproduction events are synchronized with host reproduction, so the number

Table 1. Average monthly number of bats, number of parasites, prevalence (%), parasite load (Mean ± SE) and intensity in male (M) and female (F) of *P. kuhlii*.

Month	No. of bats (M)	No. of bats (F)	No. of parasites (M)	No. of parasites (F)	Prevalence (M)	Prevalence (F)	Parasite load (M)	Parasite load (F)	Intensity (M)	Intensity (F)
May	56	28	1505	1319	100	100	26.87 ± 4.44	47.10 ± 10.62	26.87	47.10
June	55	38	829	507	47.27	84.21	15.07 ± 1.86	13.34 ± 3.71	31.88	15.84
July	29	15	31	238	93.10	80	1.06 ± 2.05	15.86 ± 2.59	1.14	19.83
August	21	12	80	231	42.85	83.33	3.80 ± 1.34	19.25 ± 2.43	8.88	23.10
September	29	10	93	113	75.86	100	3.20 ± 1.50	11.30 ± 2.39	4.22	11.30
October	19	6	101	69	52.63	100	5.31 ± 1.34	11.50 ± 2.46	10.10	11.50
November	21	9	180	59	52.38	77.77	8.57 ± 1.83	6.55 ± 2.08	16.36	8.42

of ectoparasites is highest in pregnant and lactating bats and after the parturition the peak of parasite abundance belong to juveniles (Christe et al. 2000; Bartonicka and Gaisler 2007; Lourenço 2008). The effect of variation in the intensities of a parasitic mite (*Spinturnix myoti*) in relation to the reproductive cycle and immunocompetence of its bat host (*Myotis myotis*) has shown that pregnant females were less immunocompetent and harbored more parasites than non-reproductive females (Christe et al. 2000). There are also studies that demonstrate differences in the social contact between different segments of a species population, as well as, differences in roosting habits among bat species, populations, and sexes, can cause differences in parasite load (Lourenço 2008; Sharifi et al. 2008, 2013; Postawa and Szubert-Kruszyńska 2014).

Kuhl's pipistrelle, *P. kuhlii* (Kuhl 1817) is a small bat with a circum-Mediterranean area distribution occurring in entire southern Europe. In Iran, this bat occurs both in Mesopotamian semi-deserts and in mountainous regions of the Zagros Range (Benda et al. 2006). In the present study, we summarize seasonal variation in infestation, prevalence, parasite load and intensity of the ectoparasitic in Kuhl's pipistrelle, *P. kuhlii* in western Iran. We also aim to explore the relationship between parasite load and ratio of body mass to forearm length (W/FA) as an indicator of health condition.

Materials and Methods

Kuhl's pipistrelle *P. kuhlii* (Kuhl 1817) used in this study were mist-netted in western Iran (34° 23' N, 47° 07 'E) during May to November 2013. Immediately following capture, bats were placed in individual cloth bags (30 × 50 cm). The following data were recorded: date, sex, weight (g), forearm length (mm), reproductive stage for females by palpation (pregnant, non-pregnant) (Sharifi et al. 2008) and the number of ectoparasite specimens on the wing membranes of each individual bat, as well as the ventral and dorsal parts of the body. We spent approximately 20 seconds searching each bat for ectoparasites by blowing on their pelage. Parasites were collected using a pair of stainless steel pointed tweezers and were preserved in 1.5 ml Eppendorf tubes with 70% ethanol. The sampled parasites were identified using available resource (Stanyukovich 1997; Radovsky 2010; Hosseini-Chegeni and Tavakoli 2013).

Prevalence of parasite is expressed as the percentage of parasitized individuals, relative density or parasite load as mean number of ectoparasites per bat and intensity of parasitism as the mean number of parasites found on parasitized individuals separately for male and female. The significance of patterns of prevalence parasite among male and female of *P. kuhlii* were analyzed using Fisher's exact test. Parasite load was compared using an ANOVA and infestation intensity was compared using *t*-tests. Bat health was indicated by the ratio of body mass to forearm length (Sharifi et al. 2008) and was also compared to parasite load using the Pearson correlation coefficient (Miller 2014). The statistical program package SPSS (v. 16) (spss.software.informer.com) was used for all analyses.

Results

In total, 348 bats (230 males and 118 females) were examined for ectoparasites during May to November 2013. One species of tick (*Argas vespertilionis*, Argasidae) and one species of mite (*Steatonyssus* sp., Macronyssidae) were identified as ectoparasites on *P. kuhlii*. The mite parasites did not identify in species level due to the morphological complexity. Of the total of 5355 ectoparasites that were observed in this study, 502 were belonging to *Argas vespertilionis* and 4853 to *Steatonyssus sp.* The overall prevalence of ectoparasites on all bats was 73.48 ± 6.11%. Table 1 shows the number of bats, number of parasites, parasite load, prevalence and intensity of infestation in male and female of *P. kuhlii*.

Average prevalence of total infestation on male and female bats was 66.29 ± 8.77% and 89.33 ± 3.85%, respec-

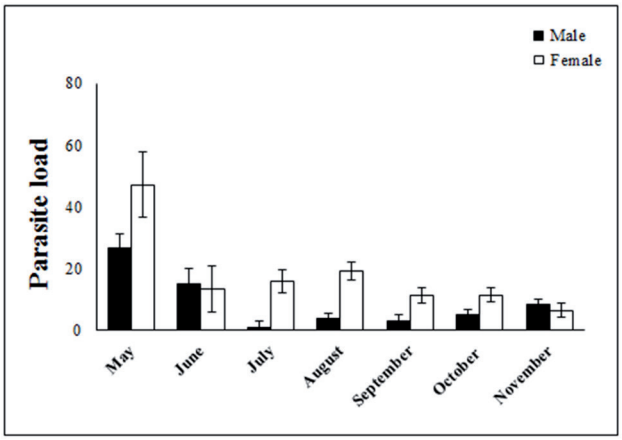

Figure 1. Variation in parasite load for males and females *P. kuhlii* in May to November 2013.

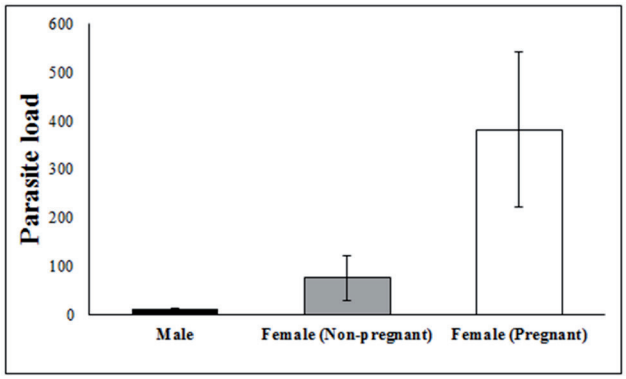

Figure 2. Total parasite load in male, non-pregnant and pregnant females of *P. kuhlii*.

tively. Prevalence was significantly different between males and females of *P. kuhlii* ($p = 0.04$). Parasite loads for males and females were 9.12 ± 3.42 and 17.84 ± 5.10 and mean intensities of infestation were 14.20 ± 4.34 and 19.58 ± 4.98, respectively. A peak of ectoparasite load recorded in May and then decrease was found until the end of November (Fig. 1). Parasite load was not significantly different between males and females of *P. kuhlii* ($p = 0.18$). Parasite intensity was not significantly different between males and females of *P. kuhlii* ($p = 0.43$). During the pregnancy until the parturitions in mid-June, a significant difference was recorded in the number of ectoparasites between pregnant females, non-pregnant females ($p < 0.05$) (Fig. 2). There was a significant relationship between parasite load and the health indicator of adult *P. kuhlii* ($p = 0.001$); (male: $p = 0.001$; female: $p = 0.13$).

Discussion

The present study provides new data regarding prevalence, intensity and parasite load in a free-living *P. kuhlii* during May to November. Average peak value for ectoparasite load was in May when the parturition had not started. Following parturition in early June a sudden decrease is seen in the ectoparasite (Table 1, and Fig. 1) in male and female *P. kuhlii.* A similar reduction in prevalence and average intensity began in June. This rapid change in the amount of ectoparasite load is the result of changes in life history traits which causes differences in the social contact between different segments of a population that can eventually cause differences in parasite load. There are many studies that support the differences in the social contact as well as differences in roosting habits that cause differences in parasite load in the temperate bats that experience a long-term winter hibernation, characterized by a rapid emergence from winter hibernacula, the formation of maternity colonies and parturition in late spring (Altizer et al. 2003; Sharifi et al. 2008, 2013; Webber et al. 2015).

Ectoparasite loads varied seasonally, declining from spring to winter. In this study, the pick of ectoparasite infestation in males and females observed in May. This is in consistent with other studies. Sharifi et al. (2008) found that the number of *Spintunix* sp. increased in June and July and decreased towards August. Observation of reproductive system in *P. kuhlii* by Barak and Tom-Tov (1991) demonstrated that hundreds of female bats aggregate at maternity colonies at the beginning of March. They reported similar group of males (7-16 individuals) in roosting sits which tend to stay in same group for 3 months. Hemmati et al. (2000) showed that in Iran *P. kuhlii* give birth in late of June. The higher host population density of bats in spring could be an important factor explaining distribution and abundance of parasites and cause of the species to face higher parasite loads (Poulin and Morand 2000; Stanko et al. 2002). Direct physical contact can exchange permanent parasites when bat species aggregate during mating or foraging (Altizer et al. 2003; Webber et al. 2015). Ticks and mite are flightless and permanent ectoparasites and host transfer depends on close contact of bats (Mathison and Pritt 2014). Age of the colony and colony size are also known to have an influence on levels of ectoparasite infestation on bats (Lučan 2006).

Our study revealed that average ectoparasite infestation was higher in females than in males, resulting in a higher parasite load in the population in spring and summer. Consistent with this study, greater prevalence and intensity of ectoparasites were found in females of little brown bats (*Myotis lucifugus*) compared to male bats (Czenze and Broders 2011; Webber et al. 2015). A similar result has also been observed between ectoparasite load in

male and female of bat fly parasites on some Phyllostomid bats in southeastern Brazil (Komeno and Linhares 1999). Results of studies conducted by Dick et al. (2003) showed that relative density of ectoparasite load in several female bats from central Pennsylvania was higher than males. In a study on grooming behavior and parasite load in the greater horseshoe bat (*Rhinolophus ferrumequinum)*, a significant difference between parasite load of male and female have been reported (Wohland 2000). It has been shown by several authors that females of most temperate zone bat species usually aggregate during the breeding season in nursery colonies, whereas males are mainly solitary or in small groups and occupy satellite roosts (Lewis 1995). The effect of such colonial habits of female bats during reproduction in favor of parasite transmission has also been revealed (Christe et al. 2000). Moreover, high temperature of nursery roost sites, including the heat generated by tight associations among individuals in a colonial cluster, may also favor parasites' reproductive output (Marshall 1981). These two factors could lead to a strong sex-linked difference in the opportunity to become infested (Christ et al. 2007). Therefore, sex bias in parasite rate most likely seems from sex-specific differences in host social behavior, with female aggregation during the reproductive period that play a central role for the evolution of this pattern.

Most biologists assume that ectoparasites on bats can influence the behavior and health of their hosts and may act as vectors for disease or cause physical damage to their hosts (Marshall 1982). However, there are other reports that have documented that ectoparasite load has no effect on health condition of host (Sharifi et al. 2008, 2013). In conclusion, in our study there was a significant relationship between parasite load and body condition of adult *P. kuhlii*.

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